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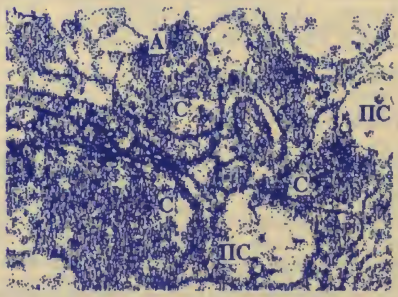
PROBLEMS OF CRYOBIOLOGY



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RENEWAL OF BIOLOGICAL STRUCTURES AND FUNCTIONS WITH THE HELP OF LOW TEMPERATURES AND CRYOPRESERVATION – A NEW TREND IN BIOLOGY AND MEDICINE

Cold is known either to preserve or damage biological systems. But is the action of cold capable of triggering genetically programmed favorable changes (renewal) in biological systems? This problem received poor attention in the literature. However, a current state of ecology, a rise in the rate of diseases and lethality testifies to its urgent nature. The authors produce new competitive technologies of increasing transplantability of hemopoietic cells, fertilizing ability of spermatozoa, efficiency of treatment of hypoxic, comatose and post-irradiation states of the organism and new methods of their diagnostics.

Biological systems may be preserved in viable states with the help of low temperatures and preservation. However, in recent years the original level of the biosystem cannot be used as a standard of its quality due to ecologic, radiation and other environmental events. The problem of survival of mankind thus urged the development of the new trend in biology and medicine – cryobiology of renewal, which deals with the mechanisms of irreversible favorable changes (renewal) in biological structure and functions with the help of low temperatures.

The idea of the qualitative renewal of the cryopreserved biosystems was strongly supported by A.M.Utevsky, biochemist and cryobiologist widely known both in the Ukraine and abroad. The basic principles of the cryobiology of renewal were closely related to his own research activity. Cryopreservation, as claimed by A.M.Utevsky, is a qualitatively new phenomenon, which is characterized not by pre-formation and return to the origin, a transition not from the inanimate to the living state, but from a specific state of the limited functional ability to polyfunctional vital activity.

The development of the basic principles of cryobiology was preceded by a long-term stage of the elaboration of the methods of identifying vital activity of the cells and diagnosis of the pathologic states of the organism, which are currently protected by more than 40 inventor's certificates. A new trend in cryocytophysiology of the cell – study of the interrelated intracellular structures and functions – has been launched. Experimental and theoretical R&Ds of the authors underwent clinical trials and were introduced into clinical practice in 15 medical establishments of the cities of Kharkov, Poltava, Lvov and Petropavlovsk. The above was completely disclosed in the monography "Cytochemistry of the frozen cell" [3] and atlas "Cytochemistry of the bone marrow during cryopreservation" [2], as well as in the article "Qualitative renewal of the cell properties after cryopreservation" [1].

Given below is a brief description of the basic R&Ds of the authors, dealing with the early pre-clinical diagnostics of the comatose, hypoxic and post-irradiation states of the organism, which are not identified by routine methods, and the ways of their treatment. For instance, the formula of the closed author's certificates №1329352 (E.I.Oboznaya, 1987) and №1822761 (S.I.Loginov, E.I.Oboznaya, 1992) describes the methods of the early pre-clinical diagnostics of the patients with ischemic, hemorrhagic and post-irradiation pathologies with the aim of their timely treatment, as well as reanimation measures to force the patient out of the comatose state, the duration of which was subsequently shortened by 3–5 days on average.

One of the tasks of cryobiology of renewal is development of new technological processes, capable of transferring biosystems to a qualitatively new level of homeostasis. There are several approaches to its solution, namely: (i) transfer of XA-genes, which induce heterosis effect, and genes of the antifreeze proteins; (ii) induction of micromutations and cryohybrids with heterosis effect; (iii) activation of the energy-forming and other systems in the stationary kinetics of biochemical responses. Currently theoretical and practical bases for these research have been developed. For instance, the expression of the XA-genes was found to take place at the level of transcription, not to be stimulated and induced by thermal stress, aqua shock or injury [7]. Expression of the XA-genes at −4 °C is coordinated and exercised at the level of transcription. The genes, the expression of which is induced at low temperatures (Kondo K., Inouye M., 1991), were identified in the yeast *Saccharomyces cerevisiae* by a method of differential hybridization. The analysis of the sprouts of *in vitro* translation of poly $(A)^{+}$RNA, isolated from various plant tissues, testifies to a transspecific expression of genes, induced by low temperatures (Danyluk J., Sarhan F., 1990). It is believed that development of freeze-tolerance is associated with the expression of specific genes, regulated at the level of transcription. Cold hardiness of wheat, for example, is controlled over by 2 loci of a 5A chromosome [8]. One locus, which partially controls cold hardiness, is closely linked with the locus, responsible for the length of the leaves. The authors suppose that the other locus is closely connected with a $V_{rn}1$, providing for the way of living. This locus may partially control a rosette-like type of the bush. It was assumed that a $V_{rn}1$ gene is dependent on the temperature and represents, to some extent, a triggering mechanism of vernalization, cold hardiness and development of a rosette-like type of the bush. In other words, a $V_{rn}1$ gene may encode the synthesis of proteins, which are resistant to low temperature. A gene of the wheat resistance to low positive temperatures (Whelan E.D.P., 1991) was isolated. This gene is predominant and localized on a short shoulder of a *6 D Chinese spring* chromosome. Molecular cloning was conducted, and the new genes were characterized, which are responsible for the cold hardiness in rice (Bink Le Tran, Oono Klyohary, 1992). In natural environment most of 40 genes of the antifreeze proteins, isolated from fish oviducts, reside in the sections of DNA with the length of 708 pairs of bases, alternating as tandemly direct repetitions [9]. Every repetition contains a single gene of the antifreeze protein with the length of $1x10^3$ pairs of bases, and transcription orientation is similar to that of other genes. It was found that genes of the antifreeze proteins are tandemly connected and grouped in the flat-fish genome. A study of the genome organization showed that formation of the antifreeze polypeptides relates to the family of genes, which appeared later in the process of evolution, as compared with the rest of the genome (Shidio Sond et al., 1987). The authors also observed the expression of the cold shock proteins (CSP), which is encoded by specific genes: the synthesis of 14 proteins increases in *E.coli* during low temperature stress, while the synthesis of the thermal stress proteins is rapidly reduced (Van Bogelen et al., 1992). A subunit A of the DNA-gyrase is a cold shock protein, and it goes on synthesizing after a transfer of the growing cultures *E.coli* from 37 C to 10 °C (Jones Pamela G. et al., 1992). A *hns* gene, which is positioned on 27 min of the *E.coli* chromosome and which encodes a H-NS nucleotide protein with a dimension of 15.4 kD, was found to belong to the regulators of the cold shock (Anna La Teana et al., 1991). The authors demonstrated the influence of the low positive temperatures on the inheritance of the duration of the life period of the *D.Melanogaster* mature species (Gonemura Isamu, 1990). When varying the temperature in the range of 17–27 °C the autosomic alleles A1 and A_2 of the duration of the life period exhibited major effects: prolongation of the life period with the lowering of temperature. Sex-bound

alleles of the duration of the life period X_1 and X_2 are of secondary importance in their effect on the life period duration. The genes of resistance to low (12 °C) temperature were studied in the groups of *Poecilia reticulata* with respect to the sex-bound inheritance (Fujio, 1990). It was assumed that the allele of resistance (R) to low temperature is predominant with respect to the allele of susceptibility (t); here a R_2 gene, apparently, is linked with a sex X-chromosome. It was reported that the level of expression of R_2 gene experiences a severe environmental (physiological) control, thus providing for a considerable differentiation of its manifestation in various lines of the groups, supported by other researches: the duration of the incubation life period of the winter flat-fish relates to the temperature of development and parental action by 93 and 3%, respectively (Chambers D.Ch., Leggett W.C., 1989). The authors also observed a high inheritance of the resistance to low negative temperatures (0, −1, −8 °C) in young sprouts of rice (Morishima, Hiroko et al., 1991) and clover (Garadua S.R. et al., 1990). The authors believe a transfer of the XA-genes may increase frost resistance and yield capacity of plants. A method of transfer of the antifreeze protein genes may increase cold resistance of some fish.

Induction of cryomutations and cryohybrides with a heterosis effect. The authors demonstrated that application of particular modes of cryopreservation of hemopoietic cells at −196 °C permits to purposefully alter morphofunctional properties and obtain mutants with a heterosis effect (E.I.Oboznaya, O..Markova, 1979). The mutant megakaryocytic forms of the cryopreserved bone marrow were identified. The latter are characterized by a rise in the metabolically active zone, described by M.I.Korenevskaya et al. (1982), which is used for construction of a polysaccharide membrane components of future platelets and intensification of platelet separation. Five-ten years later the heterosis effect, induced by cryocooling, was reported by other authors. It was found that particular modes of treatment of yeast microorganisms with supralow temperatures (−180÷−190 °C) make it possible to purposefully alter morphofunctional properties and to obtain mutants with the useful biochemical and technological properties (C.Ts.Kotenko et al., 1984). A biologically active yeast strain (M-12x) with the altered and increased morphology was isolated, which is capable of fermentation at relatively low (−11÷−12 °C) temperatures, improving the quality of wine materials due to a higher synthesis of endogenous antioxidants, lower synthesis of aldehydes and volatile acids. A heterosis cryohybrid of the German carp and wild carp was bred, characterized by a growth rate, which is by 2-fold higher as compared with parental forms (E.F.Kopeika et al., 1990). The genes, affecting the heterosis effect, were localized. The greatest contribution into a heterosis effect of the cold mutant strain *D.melanogaster* (higher reproductive ability) was made by a chromosome 3, then comes 2, 1 (Guerra D., Cavicchi, 1984). Similar experiments may be conducted using various biological systems owing to the greatest discovery, made by geneticists. A certain universal nature of the code was confirmed. It was shown that similar triplets encode similar amino acids in the cell-free systems of various organisms (bacteria, mammalian cells). This code turned out to be universal for viruses, algae and sea urchin. Human genes represent various sequences of one and the same four letters of the "genetic ABC", which comprise the genes of fish, cereals, bacteria and yeasts, namely: adenosine, cytosine, guanine, thymine.

Activation of energy-generating and other systems in the time course of stationary kinetics of biochemical responses. The authors investigated the mechanism of cryoinjury, in which a leading role is played by the outflow of a cytochrome *c* out of the mitochondria and reduction in the activity of the respiratory enzymes (E.I.Oboznaya, O.P.Markova, 1976). Ten-twenty years later the data were supported by other authors (Mory Yoki et al., 1986; A.Yu.Petrenko, 1993). It is known

that the bulk of the energy of biological oxidation is released as a result of the responses, catalysed by the cytochrome system, and reserved (stand-by) capabilities of the respiratory enzymes are very high. Under physiological conditions cytochrome oxidase manifests only 5% of the maximum catalytic activity. Activation of the energy-generating system in the time course of the stationary kinetics is possible due to the existence of two forms of the respiratory chain – activated and inactivated, which are transformed into one another, and the ratio between which is regulated by the concentration of oxygen in the medium or by the amount of the cytochrome *c* in the cell. The early lack of these substances results in the activation of the energy-generating systems. This state becomes highly stable when the mode of the deficit of oxygen or cytochrome *c* is repeated several times. Activation of the enzymes of the respiratory chain, which provides for a high rate of the electron transport (Atkinson D.E., 1968; E.M.Khvatova et al., 1977), serves as the molecular basis for a rise in the bioenergetic activity of the mitochondria. The development of the activated form of the respiratory chain may result from the change in its conformation during transfer of electrons. Using a kinetic model the authors confirmed the transfer of electrons along the respiratory chain to be accompanied by the development of a more compact form of the latter, which is characterized by a rise in the resistance to lytic factors (V.V.Kupruyanov et al., 1974). The experiments conducted have shown that a short-term response of the rat liver mitochondria during cold exposure (2–4 °C) involves a rise in the concentration of the cytochromes or acceleration of their synthesis, or slowing down of their decay (E.I.Mokhova, I.V.Zhigacheva, 1977). As the cold grows deeper, a rise in the cytochromes increases the oxidative exchange, thus resulting in the intensification of the mitochondriogenesis. After one month of cold exposure the concentration of the cytochromes increases, and the mitochondria with the new properties are developed. This provides for a stable acceleration of the rate of oxygen consumption by the liver.

Thus, the authors present a theoretic and experimental substantiation of the renewal of biological structures and functions by means of activating the energy-generating systems in the stationary time course of kinetics for the electron transfer, which underlies the authors' development of the new technologies of increasing the quality of cryopreserved animal and plant biosystems. According to the claim of the patent N1441504 of the Russian Federation (E.I.Oboznaya, A.B.Savchik, 1994) the cells of the bone marrow and embryonic liver, recovered after cryopreservation at 4 °C, exceed the control levels as to the activity of the succinate dehydrogenase (SDG) by 2- and 3-fold, respectively. The SDG activity of the cryopreserved hemopoietic cells, harvested from mature donors, is maintained up to 10 days (1 day in control); when the donors were embryonic, this was as high as 21 days (2 days in control). The data were clinically supported: the acute period of the irradiation disease following transplantation of the cryopreserved bone marrow of mature and immature mice is survived by 36 and 64% of animals as compared with 32 and 54% in control (V.I.Strona, 1978; L.P.Ignasheva et al., 1982; A.N.Goltsev, A.A.Tsutsayeva, 1984), and 72% against 29% (L.I.Simonova, 1969). The authors believe that a high clinical efficiency of the hemopoietic cells, harvested from embryos and mice, is due to their following morphofunctional features: (i) imperfection of the intracellular immune components, due to which the secondary disease either does not occur at all, or is developed much less actively at later terms. The hemopoietic cells, harvested from mature donors, are characterized by well developed immune components, thus facilitating the development of a "transplant versus host" response, which results in the death of the recipients; (ii) higher portion of the stem hemopoietic cells (SHC) and progenitor cells, which are capable of restoring hemopoiesis at earlier terms; (iii) predomination of the

erythroid cells in the myelogram (up to 90%), which are characterized by a glycolytic type of exchange. In the myelogram of hemopoietic cells, harvested from mature donors, the myeloid cells with the oxidative type of exchange are predominant, and the key enzymes of this exchange are easily injured during cryopreservation. Though macrophages are formed due to the glycolytic cycle, a persisting accumulation of the superoxidized products facilitates autolytic processes, membrane lysis, death of the cell (necrosis) and death of the organism as a whole; (iv) production of hemoglobin of a fetal type (hemoglobin F), which differs from hemoglobin of a mature type (hemoglobin A) by a higher affinity to oxygen and the ability to bind it more effectively (G.I.Kozinets, E.D.Goldberg, 1982). Hence, hemopoietic cells of the immature donors experience lower formation of the superoxidized products, thus reducing and delaying necrosis; (v) availability of the megaloblastic path of hemopoiesis, which is characterized by accumulation of hemoglobin F, the advantages of which are given above; (vi) availability of the Bessis islands (aggregation of the erythroid cells around the macrophage). This phenomenon was duly studied. The investigations conducted showed that 34% of the cells, carrying a $F_{4/80}$ marker of macrophages, express a EPO gene (Wght Ch. et al., 1989). It was concluded that in the normal hemopoiesis the macrophages activate the gene of erythropoietin, while erythropoietin effectively mobilizes SHC for their transplantations: experimental injection of erythropoietin (500 units/kg) to young pigs sharply increased the yield of SHC (Smith D.M. et al., 1993). The authors revealed the role of erythropoietin during low temperature exposure with respect to another type of the cell death – apoptosis (programmed death of cells). Cooling of the BWS 147 thymomic cells down to 0–2 °C visualized fragmentation of intranucleosomic DNA (Kruman J. et al., 1992). The experiments on *in vitro* injection of ^{3}H-thymidine demonstrated ruptures in the DNA of the erythroid progenitor cells (Kounry M., Bondarant M., 1990). In the absence of erythropoietin the erythroid progenitor cells accumulated split-off fragments of DNA: in 2–4 hours they developed apoptosis features, and in 16 hours they died. In the presence of erythropoietin the ruptures in DNA were reduced by 2.7-fold, erythroid progenitor cells survived and were differentiated into reticulocytes. Thus, the revealed six morphofunctional distinguishing features of hemopoietic cells, harvested from immature donors, are mainly due to their ability to postpone necrosis and apoptosis, as well as to mobilize SHC for their transplantation.

According to the formulae of the patent N 1822761 of the Russian Federation (S.I.Loginov, E.I.Oboznaya, 1994) and patent N 5225 of the Ukraine (S.I.Loginov, E.I.Oboznaya, 1995) (in teamwork with M.I.Kramar), it was found that after 2–3 days of hypothermic storage of spermia the activity of SDG increased in the immature and mature cells by 30 and 32%, respectively. A drawback of this technology is a stimulating effect of short duration. In cooperation with T.F.Stribul the authors managed to gain a stimulating effect of cryopreservation (–96 °C) at the stage of germination of seeds with a subsequent rise in the soy-bean plant biomass. The new technologies, which are currently developed by the authors, involve a principle of the programmable activation of the morphofunctional properties of the cooled and cryopreserved biosystems in the stationary time course of kinetics of biochemical responses, thus providing for a higher level of homeostasis.

At the beginning of 1995 the article "Cryobiology of renewal: facts and prospects" (E.I.Oboznaya-Pechenezhskaya, V.I.Grischenko, E.Ya.Pankov [4]) was forwarded for reviewing to independent foreign reviewers. The experts concluded that cryobiology of renewal is a promising trend in biology and nature, and is a prerogative of its authors. The results of their own investigations nicely fit a conceptual model of renewal by cold. For instance, V.I.Baschenko et al. (Russia, St.-Petersburg, Scientific-Research Lab of the Centre of Blood and Tissues of the

S.M.Kirov Military Medical Academy), when studying the state of the supraspiral DNA of the "nucleotides" of the bone marrow cells, found a decondensing function of cryopreservation. Decondensation is known to involve constitutive genes and to provide for a possible realization of the mechanisms of the operon regulation, which underlie genetically programmed renewal. E.Siebzehnrubl (Germany, Erdangen-Nurenberg, Women Hospital of the School of Obstetrics) believes cryopreservation of the fertilized human oocytes increases probability of pregnancy development in the cycles of fertilization *in vitro* and when grafting embryos. E.J.Kendall (Canada, Institute of Plant Biotechnology) claims that development of frost hardiness in summer wheat plants is related to eight unique polypeptides (which he discovered by means of a biochemical analysis) and a higher level of sucrose. Terry D.Beacham (Canada, Pacific Biological Station), using 17 enzymatic systems, encoded by 23 loci, found that survival of embryos and young off-spring of humpbacked salmon of male and female lines and their development at low temperatures (4 and 15 °C) do not relate to heterozygosity of the producers. In other words, temperature factor plays a leading, genetically programmable role in the process of survival of biosystems. A reviewer Charles Guy (USA, Florida, Institute of Crop and Agricultural Investigations) in his works gives a review of the data on genetic aspects of frost hardiness and cold adaptation (CA). Allan K.Hardacre (New Zealand, Institute of Food Cultures) identified a genetically programmed variability in frost hardiness of maize sprouts. K.Ebine (Japan, Tokyo, Cardiological Centre) reported on a high resistance of cryopreserved hemopoietic cells to the virus of hepatitis: transfusion of unfrozen whole allogenic blood became the reason for the development of hepatitis in 13.7% of cases (49 out of 357 patients), while after transfusion of the frozen allogenic blood posttransfusion hepatitis was not developed at all (0%). D.E.Pegg, Editor-in-Chief, *Cryobiology*, also gave a positive assessment of the hypothesis of a qualitative renewal of cryopreserved cells and tissues. A first step to cooperation was made. K.Ebine (Japan) offered to publish the article in question in the journal "Low Temperature Medicine" (LTM), of which he is the editor. Ernst Siebzehnrubl (Germany) became a subscriber to the journal "Problems of Cryobiology". Both of them produced a minor financial support to investigations in the field of cryobiology of renewal.

There exist several paths of a qualitative renewal of biosystems using low and supralow temperatures. One of them is a rise in the potential statistical frost hardiness, since under natural conditions or when using agrotechnical factors it is not realized (E.M.Poltarev et al., 1991). Indeed, there were reported, e.g., an increased reparative ability and growth rate in the hybrids of the heterosis maize sprouts following exposure to supralow temperatures (–196 °C), though they did not demonstrate an increased cold hardiness (V.G.Shakhbazov et al., 1973). The kinetic investigations have shown that in the frost-resistant winter wheat the new proteins with a high molecular mass were found in 48 hours after the start of the cold treatment, i.e. long before they demonstrated a maximum resistance to frost (Abromeit Midori et al., 1992), which was supported in other systems: duration of the incubation of the winter flat-fish is determined by the temperature of development and parental influence by 93 and 3%, respectively (R.Chambers et al., 1989). When changing the temperature in the range of 17 to 27 °C the autosomic alleles of the life period duration (LPD) of mature *D.Melanogaster* A_1 and A_2 demonstrated the effect: prolongation of LPD with lowering of temperature (Y.Fujioet al., 1990). Sex-bound LPD alleles X_1 and X_2 play secondary roles in affecting the duration of life period. It is thought that a gene of resistance to low temperature (12 °C) is bound with a sex X-chromosome, and the level of its expression experiences a considerable environmental control.

Another trend in the qualitative renewal of biosystems is an increase in the resistance of biosystems to irradiation, induced by low and supralow temperatures: when the ovarian fibroblasts of the Chinese hamster and microorganisms were X-rayed, temperature lowering from 22 °C down to −196 °C resulted in the reduction of the chromosomal injuries and increased the cell survival by 3.5-fold [5]. A resistance to irradiation in lethal doses at −196 °C in the hemopoietic stem cells (CFU-S) was by 3.45-fold higher, and in the progenitors of the granulocyto-macrophagal cells (GM-CFU-C) of bone marrow – by 3.04-fold higher as compared with the normothermic conditions (Liu Znobin, 1989). On the whole, at temperatures below −100 °C the mammalian cells become more resistant to cooling as compared with room temperature (22 °C). These data were supported in plant biosystems: in the first hours after irradiation, i.e. in the phase G_2 at 34 °C a lot more *Vicia Faba* cells are injured as compared with 20 °C, and at 4 °C the amount of the cells with aberrations is even smaller (N.N.Eliseyenko, M.V.Maksimova, 1975). A third trend involves a decrease in the occurrence of viral hepatitis using low and supralow temperatures (G.Echert, 1974; V.A.Agronenko et al., 1977; H.J.Altor, 1978, K.Ebine [6], etc.).

Genetically programmed renewal of biological structures and functions with the help of low temperatures is a natural phenomenon: peroxidases from the tissues of the northern plants demonstrate maximum activity at the temperatures which are considerably lower than the temperature optimum for enzymes of the plants of other regions (V.G.Alekseyeva, 1990).

The most important discovery of the leading role of low temperatures in the interlinked relations "genotype-environment" forces us to revise the early knowledge on the selective property of low temperatures, which were related solely to selection of resistant forms due to elimination of the irresistant ones.

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ОБНОВЛЕННЯ БІОЛОГІЧНИХ СТРУКТУР І ФУНКЦІЙ ЗА ДОПОМОГОЮ НИЗЬКИХ ТЕМПЕРАТУР І КРІОКОНСЕРВУВАННЯ — НОВИЙ НАПРЯМОК В БІОЛОГІЇ ТА МЕДИЦИНІ

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Відомо, що холод зберігає або пошкоджує біооб'єкти. Але чи може холод викликати генетично запрограмовані сприятливі зміни (обновлення) в біооб'єктах? В літературі це проблема майже не обговорювалася. Однак її актуальність при сучасному стані екології, зростанні захворюваності та смертності очевидна. Авторами представлені нові конкурентноспроможні технології підвищення трансплантаційної здатності гемопоетичних клітин, запліднюючої здатності сперматозоїдів, ефективності лікування гіпоксичних, коматозних і постпроменевих станів організму та нові методи їх діагностики.

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ОБНОВЛЕНИЕ БИОЛОГИЧЕСКИХ СТРУКТУР И ФУНКЦИЙ С ПОМОЩЬЮ НИЗКИХ ТЕМПЕРАТУР И КРИОКОНСЕРВИРОВАНИЯ — НОВОЕ НАПРАВЛЕНИЕ В БИОЛОГИИ И МЕДИЦИНЕ

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Известно, что холод сохраняет или повреждает биообъекты. Но может ли холод вызвать генетически запрограммированные благоприятные изменения (обновление) в биообъектах? В литературе эта проблема почти не обсуждалась. Однако ее актуальность при современном состоянии экологии, возрастании заболеваемости и смертности очевидна. Авторами представлены новые конкурентноспособные технологии повышения трансплантационной способности гемопоэтических клеток, оплодотворяющей способности сперматозоидов, эффективности лечения гипоксических, коматозных и постлучевых состояний организма и новые методы их диагностики.

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ON THE MECHANISM OF REGULATION OF PERMEABILITY OF BLOOD-BRAIN BARRIER OF THE COOLED BRAIN. Report 4. Ultrastructural peculiarities and functional activity of blood-brain barrier during rhythmic cold exposures

The authors investigated the ultrastructure of the blood-brain barrier (BBB) and synaptic apparatus of rats during hypothermic treatment. They studied secretion, reception and permeability of BBB to monoamines. Rhythmic cold exposures were shown to increase by 3–4-fold the BBB permeability to ^{3}H-norepinephrine, but not to ^{3}H-serotonin. The electrongrams indicate that following cold exposures the synaptic apparatus is in the state of tension, while the endothelium of the microvessels demonstrate clatrin vesicles, and the processes of receptive transcytosis are activated. The neurotransmitter processes, which determine the BBB permeability, may be a trigger neurophysiological mechanism of changes in the central thermal regulation during hypothermic treatment.

A study of the fine structure of the BBB elements of the thermoregulatory centers of hypothalamus and changes in its permeability to neuromediators during cold exposures seems to be urgent enough for solving the problems of cryobiology, related to the investigation of the role of the BBB in the mechanisms of maintenance of the temperature homeostasis in mammals. Still, a review of the literature on this matter testifies to the absence of complex studies of both physiological and ultrastructural characteristics of the BBB during hypothermia. Hence, the aim of the present study was to investigate the ultrastructural basis of the BBB per-

meability of the hypothalamic thermoregulatory centers to monoamines during rhythmic cold exposures.

White male rats with the body weights of 180–200 g were used in experiments. Rhythmic cold stimulations of the caudal thermoreceptors (PPP) with the 1-sec duration and 0.1 Hz frequency (0.1 PPP) were conducted by means of cooling the tail of the animal in the iced (–5 °C) water in the automatical mode. The fine structure and functional peculiarities of the BBB were studied in a mediopreoptical region of the anterior hypothalamus and mamillary and premamillary nuclei of the posterior department of hypothalamus. In different groups of animals the 0.1-PPP treatments were conducted for 20, 40, 60 and 80 min. The material for neurophysiological and electron-microscopic investigations was collected immediately after closing the exposure, and in 6, 10, 14, 18 and 60 hours after treatment. The animals were narcotized by the intraperitoneal injections of the mixture of sodium thiopental and sodium oxibutyrate in a dose of 30 mg/kg and 100 mg/kg of the body weight, respectively. Heparin and iproniazidum were injected intraperitoneally at a dose of 500 units and 1 mg per 100g of the animal body weight, respectively. The blockade of α and β-adrenoreceptors was induced by perfusion of *ventriculus tertius* by 2μM of phentolamine solution and propanolol and intraperitoneal injections at a dose of 1 mg per 100 g of the animal body weight. Heparin and iproniazidum were intraperitoneally injected at a dose of 500 units and 1 mg per 100 g of the animal body weight, respectively. A study of the secretion and permeability of the BBB of the brain to tritium labeled norepinephrine (^{3}H-NE) and serotonin (^{3}H-ST) were performed by a radioisotope method using a *push-pull* cannula [2, 9]. The hypothalamic tissue was prepared for electron-microscopic investigations in a routine manner [5]: use was made of the electron-dense markers – horse-radish peroxidase (according the method of Graham and Karnovsky [13]) and 2%-solution of lantane nitrate intravenously.

Morphometric and stereometric analysis of the electron-microscopic photographs were conducted with a MORPHO-TOOLS computer system. The following features were calculated: (i) the ratio of the mean area of the endothelial vesicles to the mean area of the cytoplasm of the endotheliocytes of the hypothalamic capillaries; (ii) the ratio of the mean length of the abluminal and luminal depressions to the mean length of the abluminal and luminal contours of the endotheliocytes; (iii) the ratio of the mean length of the microvilli to the mean length of the luminal contour of the endotheliocytes; (iv) the ratio of the mean area of the basal membrane to its mean perimeter; (v) the ratio of the mean area of the endotheliocytes to their mean perimeter; (vi) the ratio of mean length of the active zone of the synapses to the mean length of the synaptical contact.

The electron-microscopic analysis of the studied samples testifies to the fact that the hypothalamus of the rat possesses a complex microscopical organization and it is represented by the nervous and glial cells with numerous processes, which form the neuropile, filled-in with a network of the terminal vessels. The vascular endothelium with the basal membrane and pericytes, as well as the processes of the astroglia, surrounding the capillaries, are currently believed to comprise a morphological basis of the blood-brain barrier [6, 8, 9]. That is why the authors paid particular attention to the ultrastructural rearrangements in these very basic elements of the BBB.

It was found that already in 20 min after the start of 0.1 PPP there occur certain changes in the submicroscopic organization of the studied regions of hypothalamus, which testify to the activation of various groups of cells. A part of the neurons, residing in the vicinity of the blood vessels, demonstrate reactive alterations: the saturation of the matrix and many organelles increases, a Golgi complex is saturated, resulting in the formation of the bubbles and granules, and there

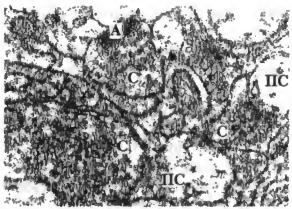

Fig. 1. Active synapses in the anterior department of the rat hypothalamus (40 min of 0.1 PPP), X40000: С – presynaptic bud with synaptic bubbles, ПС – postsynaptic bud, А – active zone of synaptic contact

appear lysosomes (frequently their shapes are irregular) and numerous mitochondria and ribosomes. The synaptic apparatus is in the state of the increased functional pressure: the synaptic bubbles in many synapses aglutinate with one another and fuse with the synaptic membrane, and their amount is subsequently reduced; the extent and electron density of the zone of the synaptic contacts increase, and the mean length of the active zone is raised (Table 1, Fig. 1, 4). It should be noted that the disclosed alterations in the fine structure of the neurons are most vividly manifested in the mediopreoptical region of the anterior hypothalamus (as compared with the posterior one), in the region of which nervous cells are known to be located, which are activated during temperature exposures [3]. Thus the disclosed reactive alterations of the part of the neurons may indirectly support their belonging to the pool of thermocompetent cells.

Table 1

Morphometric analysis of the electron microphotographs of the synapses of the anterior department of hypothalamus of rats during 0.1 PPP

Objects of investigations	Control	Time of exposure, min			Time after exposure, h				
		20	40	60	6	10	14	18	60
Relative length of the active zone of the synapse	0.4± 0.04	0.6± 0.03	0.55± 0.04	0.5± 0.03	0.49± 0.04	0.41± 0.03	0.39± 0.04	0.4± 0.03	0.4± 0.06
Amount of the postsynaptic bubbles	65±5	28±4	33±4	40±5	38±5	60±6	64±6	66±7	65±5
Amount of the active synapses, %	55±5	90±9	80±7	76±8	74±7	55±6	57±6	58±6	55±7

Rhythmic cold exposures exercise a strong action on the fine structure of the astroglia, especially with respect to the processes of the astrocytes, surrounding the terminal vessels. In 20 min after the start of the exposure the electron density of the terminal limbs is reduced, which to some extent resembles edema, disappearing by the 80th min of exposure (Fig. 2). Many researchers believe that it is just astrocytes which facilitate maintenance of a particular ultrastructural organization in the endothelial cells of the brain capillaries, which provide for the BBB functioning. It is supposed that the astrocytic glia induces formation of dense contacts, specific enzymes of the BBB, and inhibits pinocytosis, formation of the endothelial channels and fenestration [8, 14]. If it is indeed so then the reported reactive changes in the astrocytes during 0.1 PPP should result in the corresponding rearrangements in all the basic ultrastructural elements of the BBB.

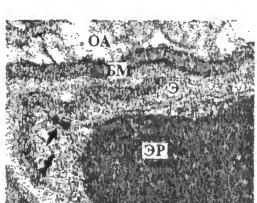

Fig. 2. Pinocytic vesicles (arrows), containing horse radish peroxidase, in the cytoplasm of the endotheliocyte (Э) of the capillary of the mediopreoptical region of the anterior hypothalamus of the rat (60 min of 0.1 PPP). X60000: БМ – basal membrane, ОА – astrocyte processes, ЭР – erythrocyte in the capillary lumen

Indeed, the most prominent changes are identified in the fine structure of the endotheliocytes, their dense contacts and the basal membrane, which surrounds the vessel. By the 20th min of exposure the relative

area of the basal membrane is significantly (by 3–3.5-fold) reduced. By the 40–60th min this index increases, being by 30–40% below the normal level. It should be noted that in the capillaries of the anterior department of hypothalamus the relative area of the basal membrane is reduced by 30–40% higher as compared with the capillaries of the posterior department. These changes persist for at least 18 hours after a 60-min exposure (Table 2).

Table 2

Morphometric analysis of electron microphotographs of the capillaries of the anterior department of hypothalamus of rats during 0.1 PPP

Objects of investigation	Control	Time of exposure, min			Time after exposure, h				
		20	40	60	6	10	14	18	60
Relative area of endotheliocytes	0.1± 0.02	0.15± 0.01	0.15± 0.02	0.1± 0.02	0.11± 0.03	0.12± 0.02	0.1± 0.03	0.1± 0.03	0.1± 0.03
Relative area of basal membrane	0.17± 0.02	0.05± 0.005	0.14± 0.01	0.11± 0.02	0.11± 0.02	0.15± 0.03	0.11± 0.01	0.12± 0.03	0.17± 0.01
Relative length of depression of abluminal membrane	0.045± 0.01	0.1± 0.05	0.043± 0.01	0.05± 0.01	0.06± 0.01	0.065 ±0.01	0.04± 0.01	0.07± 0.01	0.05± 0.002
Relative length of depression of luminal membrane	0.076± 0.01	0.18± 0.02	0.22± 0.02	0.2± 0.02	0.2± 0.03	0.17± 0.03	0.14± 0.02	0.16± 0.02	0.08± 0.004
Relative area of microvilli	0.04± 0.008	0.1± 0.02	0.15± 0.03	0.07± 0.01	0.07± 0.01	0.06± 0.01	0.04± 0.008	0.05± 0.009	0.04± 0.003
Relative area of endothelial vesicles	0.0075 ±0.001	0.013± 0.001	0.014± 0.002	0.026± 0.003	0.025 ±0.003	0.008 ±0.001	0.01± 0.001	0.01± 0.001	0.008 ±0.002
Amount of trimmed vesicles, %	0	40±5	29±5	67±7	60±7	0	30±4	33±4	0

The gel of the basal membrane, formed by glucosamine glycanes, builds up molecular and electrostatic filters, which provide for a selective permeability of the membrane to negatively charged molecules. Besides, proteoglycanes possess osmotic activity, which also regulates permeability [4]. This allows to consider the basal membrane as an additional regulator of the BBB functional activity [8]. The authors believe a reduction in the area of the basal membrane may reflect the extent of polymerization of glucosamine glycanes and affect the level of the BBB permeability. The relative area of the endothelial cell changes in the opposite manner during 0.1 PPP. The first 20 min of exposure witness a 1.5-fold rise in the relative area of the endotheliocyte as compared with control. Such a rise is maintained for another 20 min of cooling, returning to the normal values by the 60th min of exposure. An increase in the relative area of the endotheliocyte in the posterior region of hypothalamus is higher (by 33%) as compared with the anterior department (Table 2).

Considerable variations during 0.1 PPP are found within the surface of the endotheliocyte (Fig. 3, 4). Numerous depressions are developed at the luminal and abluminal contours, the length of which by the 20th min by 2-fold exceed the control values, staying at a heightened level for at least 18 hours after exposure (these changes are more pronounced in the anterior department of hypothalamus). It should be noted that during 0.1 PPP the amount of the microvilli on the luminal surface of the endotheliocyte increases, the relative length of which is prolonged by more than 2-fold. Such a rise persists for 10 hours after exposure, returning to the normal values by the 12–14th h (Table 2).

The fine structure of the nucleus and cytoplasm of the endothelial cells during 0.1 PPP is close to the normal one. The activization of the mitochondria is reported, which is manifested in the enlightening of the matrix and increase in its area. A characteristic feature is the appearance of a large amount of the endocytic vesicles of various dimensions in the capillary endothelium of the anterior and posterior

departments of the hypothalamus by the 20th min of exposure. Their relative area increases by 3–4-fold by the 60th min as compared with control, and returns to the normal values in 10–12 h after exposure (Table 2). Apparently, the observed surfacial changes in the endotheliocytes, as well as a rise in the amount of and the relative area of the endocytic vesicles testify to the development of the processes of pinocytosis in the endotheliocytes of the hypothalamic capillaries. The electron microphotographs reflect all the stages of the formation of the pinocytic vesicles, starting from invagination of the luminal membrane (1), formation of the open and then closed vesicle, attached to the luminal membrane, appearance of free pinocytic bubbles (20, their merging (3), formation of the channel out of the merged vesicles, ending up in the attachment of the vesicles to the abluminal membrane (4) or transformation of the vesicles in a GERL system (Golgi complex, endoplasmic reticulum, lysosomes) with subsequent recycling of the membrane (Fig. 3, 4). The above changes in the fine structure of the hypothalamic BBB elements should supposedly result in the increase in its permeability, and the reported peculiarities of the structural rearrangements in the anterior and posterior departments of the hypothalamus may correspond to various levels of permeability of the barrier. The following series of experiments were aimed to support the assumptions.

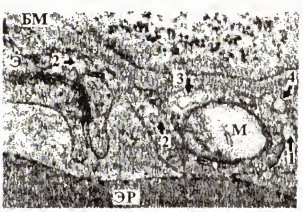

Fig. 3. Expansion of the intercelluiar contact of endotheliocytes with formation of the "funnei". The main stages (1–4) of pinocytosis in the endothelial cell (Э) of the capillary of the posterior department of the hypothalamus of the rat in 18 h after 0.1 PPP. X104000: 1 – invagination of the luminal membrane of the endotheliocyte, 2 – formation of free pinocytic vesicles, 3 – merging of pinocytic vesicles, 4 – open vesicle in the abluminal part of the endotheliocyte; БМ – basal membrane, ЭР – erythrocyte in the lumen capillary

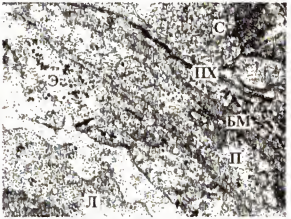

Fig. 4. Fragment of the wall of the capillary from the preoptical region of the anterior hypothalamus of the rat (60 min of 0.1 PPP). X100000. Microtubules (big arrow) and coated vesicles (small arrows) in the cytoplasm of the endotheliocyte (Э). П – pericyte, БМ – basal membrane, ПХ – precipitation of the electron-dense pellet of the horse radish peroxidase reaction among the processes of the glial cells, С – synaptic bubbles in the presynaptic bud of the axo-axonal synapse

For evaluating the BBB permeability during 0.1 PPP the authors have conducted two series of experiments using intravenous injection of the electron-dense markers – horse radish peroxidase and lantane ions. The electron-microscopic analysis of the ultrathin slices of these hypothalamic samples testify to the fact that horse radish peroxidase, while being a marker of pinocytosis, was found in the form of the electron-dense inclusions within vesicles (Fig. 2) and in the cytoplasmic channels, which were possibly formed by fusion of such vesicles (Fig. 3). Besides, sedimentation of the homogenic electron-dense pellet, formed by horse radish peroxidase and lantane ions, was partially evident behind the basal membrane between the terminal limbs of the astrocytes (Fig. 4). The experiment with application of horse radish demonstrated a rise in the electron density of the capillary basal membranes (Fig. 2).

The electron-dense pellet of the product of reaction of horse radish was also found in the contacts between the membranes of endotheliocytes. Horse radish

peroxidase was also found in the lysosomes of pericytes, which possess [4, 11] phagocytic activity (Fig. 4). It should be noted that in 30% of the studied capillary lumens the processes of pericytes came into contact with endotheliocytes, normally in the areas of contacts of endotheliocytes and cytoplasmic channels. Of particular interest is the fact of the endothelial cells breaking the contacts between them, thus forming a "funnel", deeply protruding into the capillary lumen (Fig. 3). In a few cases horse radish completely filled in the lumen of the intercellular contact.

Thus, rhythmic cold exposures bring about structural rearrangements in all the basic links of the hypothalamic BBB, which increase its permeability to the applied electron-dense markers. Apparently, during 0.1 PPP the "breakthrough" of the BBB does not occur, which should be accompanied by precipitation of the electron-dense markers along the whole perimeter of the basal membrane, and not partially, as it occurred in the experiments conducted (Fig. 4). That is why the reported changes in the functional activity of the BBB may bear physiological importance for the processes of the central thermal regulation. Apparently, horse radish peroxidase only visualizes the process of a selective transendothelial transfer of the biologically active compounds, being their "fellow travellers by chance".

As reported earlier (ref."Problemy kriobiologii", 1995, №3), during 0.1 PPP the BBB permeability significantly increases only to neuromediators, which mediate heat production (norepinephrine), and does not change with respect to the neuromediators, mediating the processes of heat return (serotonin) (Fig. 5). In the anterior department of the hypothalamus, where the main pool of the thermosensitive neurons is concentrated [2, 3], the permeability of the barrier was significantly higher as compared with the anterior departments, which nicely correlates with the peculiarities of the ultrastructural rearrangements during cold exposure. Thus, a 0.1 PPP treatment may possibly trigger specific neurotransmitter mechanisms, which selectively alter the BBB permeability, activating the hypothalamic centers of thermal regulation.

When speculating on the rhythmic cold exposures on the BBB permeability to monoamines, one cannot disregard the problem of the mechanisms, providing for a selective permeability to serotonin and norepinephrine. In the processes of maintaining the BBB resistance to serotonin the enzymatic barrier with involvement of monoamine oxidase (MAO) may play an important role. The latter is known [10] to be the enzyme, which catalyzes desamination of the biogenic amines. It was shown that even a single short-term cooling of the organism results in a prolonged alteration of the activity of the enzyme [10], and after termination of the low temperature exposure the MAO activity is maintained at an increased level for 4 and more days. These data correlate with the results of the authors own experiments. For example, injection of a nonspecific MAO-blocking agent iproniazidum during 0.1 PPP treatment increased the barrier permeability to serotonin by nearly 2-fold, while there was no further rise in permeability to ^{3}H-NE (Fig. 5). Hence, if the BBB resistance to serotonin may be accounted for by the availability of the enzymatic barrier (a rise in the MAO activity), then norepinephrine "evades" the action of the given enzyme. The authors believe that a rise in the specific BBB permeability to

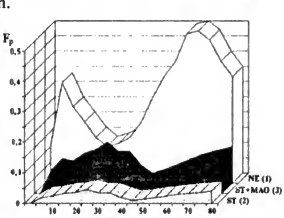

Fig. 5. BBB permeability to monoamines during rhythmic cold exposures: 1 – factor of permeability (F_p) to ^{3}H-NE in the time course of 0.1 PPP, 2 – F_p to ^{3}H-ST in the dynamics of 0.1 PPP, 3 – F_p to ^{3}H-ST in the dynamics of 0.1 PPP during MAO blockade

norepinephrine is provided for by complex structure-functional mechanisms, which were studied in the series of the latest experiments of the authors.

It is well known that initiation of the structure-functional changes, increasing the BBB permeability, may be both of humoral and neurogenic origin [3]. Predomination of one or another origin is determined by the action on the organism. For example, during hypothermic treatment the communication signals, triggering reactive changes in the BBB, are supplied, mainly, by the blood cells [1], and the authors report on the marginal elevation and adhesion of the leukocytes, monocytes, platelets to the surface of the endothelial cells. Rhythmic cold exposure did not trigger such phenomena (Fig. 4). That is why the authors assumed that a rise in the BBB permeability during such an exposure is caused by the activation of the specific neurophysiological mechanisms of the CNC. The authors managed to demonstrate the activation of the synapses of the *S*, and to a higher extent, of the *C* type already by the 20th min of exposure (Fig. 1, 4). The synaptic terminals of the *S* type contain spheric vesicles of the low electron density with the diameter of 500 A, and acetyl choline is thought to be their mediator [12]. The synaptic terminals of the *S* type, on the contrary, contain spheric vesicles of the high electron density, which is reduced after activation of the synapse. It is supposed that monoamines act as mediators in these synapses. Unfortunately, identification of the epinephrine, norepinephrine, and serotonin synapses according to their electron-microscopic characteristics is impossible today. However, the authors believe that during 0.1 PPP the noradrenergic synapses are activated, since the adrenergic synapses are very few in the hypothalamus, while neurophysiological experiments *in vivo* demonstrate a significant (by 2–3-fold) increase in a spontaneous secretion of ^{3}H-NE in the anterior hypothalamus, and an insignificant rise in the posterior hypothalamus, and secretion of ^{3}H-serotonin (^{3}H-ST) does not change (Table 3).

Table 3

Spontaneous secretion of ^{3}H-NE and ^{3}H-ST in the anterior and posterior hypothalamic departments during 0.1 PPP

Experimental conditions	Anterior department of hypothalamus		Posterior department of hypothalamus	
	^{3}H-NE	^{3}H-ST	^{3}H-NE	^{3}H-ST
Control	0.82±0.05	0.79±0.03	0.81±0.05	0.78±0.04
Anaesthesia	0.78±0.04	0.76±0.04	0.77±0.04	0.74±0.03
0.1 PPP (10 min)	2.37±0.1	0.74±0.05	1.94±0.1	0.76±0.05
0.1 PPP (20 min)	2.01±0.1	0.76±0.06	1.70±0.1	0.74±0.06
0.1 PPP (40 min)	2.63±0.3	0.77±0.07	2.09±0.3	0.71±0.07
0.1 PPP (60 min)	2.81±0.4	0.75±0.06	2.31±0.4	0.73±0.05

Application of superfusion of the hypothalamus by the blocking agents of α- and β-adrenoreceptors during 0.1 PPP reduces the BBB permeability to ^{3}H-NE down to the control level (Fig. 6). This may be indicative of the fact that a rise in the activity of the noradrenergic functional system of the hypothalamus initiates a rise in the BBB permeability.

A comparison of the results of physiological experiments and the data of the ultrastructural analysis on the development of the processes of pinocytosis in the cells of the capillary endothelium of the hypothalamus makes it possible to assume that a selective increase in the BBB permeability to ^{3}H-NE is due to the process of a highly specific adsorptive transcytosis or receptor-induced transcytosis [7]. The first stage of the specific endocytosis is the adsorption of substances on the glycocalix of the plasmalemma [7]. Exposure of the mucopolysaccharide level of the glycocalix to the intraperitoneal injection of heparin results in the reduction of the increased BBB permeability to ^{3}H-NE, caused by 0.1 PPP (Fig. 6). At the next stage the cells capture ligand molecules with the help of the corresponding recep-

tors of the plasmalemma. The intraperitoneal injection of the blocking agents of α- and β-adrenoreceptors also reduces the BBB permeability to ^{3}H-NE.

Binding of the ligand (norepinephrine in the given case) with a receptor brings about membrane invaginations, which becomes deeper, rounded, and closes, so that norepinephrine is found within the composition of the specific endosome, which is called a "coated" (trimmed) vesicle. The coated vesicles are identified in the cytoplasm of endotheliocytes already at the 20th min of exposure, and by the 60th min they are found in the 70% of the studied capillary lumens, and in the anterior department of the hypothalamus their amount is by 2.5-fold higher as compared with the posterior (Table 2).

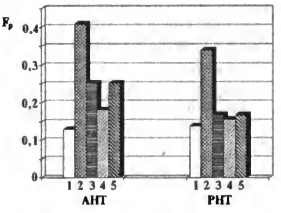

Fig. 6. BBB permeability to ^{3}H-NE during hypothermic and pharmacologic exposures (ANT, PHT – anterior and posterior hypothalamus): 1 – factor of permeability (F_p) in control, 2 – F_p by the 60th min of 0.1 PPP, 3 – F_p by the 60th min of 0.1 PPP after injection of propanolol, 4 – F_p by the 60th min of 0.1 PPP after injection of phentolamine, 5 – F_p by the 60th min of 0.1 PPP after injection of heparin

The coated vesicles on the outer surface contain clatrine "fur", which represents basket-like structures with penta- and hexagonal cells, formed due to clatrine polymerization. These organelles vary in their dimensions within one and the same cell – 50–250 nm in the diameter (Fig. 4). It is believed that a clatrine "fur" facilitates preservation of the vesicle content against lysis (norepinephrine "evades" MAO), and it is required for clutching of these organelles with the cytoskeleton and their subsequent transport in the cell. The analysis of the ultrathin slices of the hypothalamus identified most of the stages of the formation, transformation and transport of the coated vesicles in the endothelial cells (Fig. 4).

While penetrating into the cytoplasm, the coated vesicles loose clatrine cover and merges with one another or with micropinocytic vesicles (in the experiments with the electron-dense markers a "fellow traveller" – horse radish peroxidase – is captured). Bubbles of the larger size are formed, which have smooth surface, clear-contoured vesicles move within the cytoplasm along the skeleton, microtubules, in particular (Fig. 4), and are carried by NE to the abluminal surface of the endotheliocyte either directly or via transendothelial channels. It should be noted that the processes of transendothelial transfer are characterized by a seconds rhythm [4, 7]. The efficiency of the rhythmic cold exposures with a frequency of 0.1 Hz upon the BBB permeability to NE may probably relate to this fact.

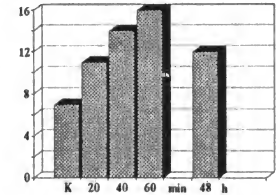

Fig. 7. Resistance to cold in the rats after 0.1 PPP. Along the X axis: K – control; 20, 40, 60 – time of exposure, min; 48 – hours after 60-min 0.1 PPP. Along the Y axis: time of active swimming in the iced water, min

The ultrastructural rearrangements of the BBB elements, caused by rhythmic exposures, persist for dozens of hours, thus providing for an increased specific permeability of the barrier. Physiological importance of a rise in the BBB permeability to NE involves, apparently, stimulation of the hypothalamic centers of thermal regulation. This is supported by a considerable rise in the resistance of the or-

ganism to cold following rhythmic stimulation of the caudal thermal receptors (Fig. 7). Thus, functional state of the blood-brain barrier should undoubtedly play a major role in the regulation of the temperature homeostasis of the organism.

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ДО МЕХАНІЗМІВ РЕГУЛЯЦІЇ ПРОНИКНОСТІ ГЕМАТОЕНЦЕФАЛІЧНОГО БАР'ЄРУ ОХОЛОДЖЕНОГО МОЗКУ. ПОВІДОМЛЕННЯ 4. УЛЬТРАСТРУКТУРНІ ОСОБЛИВОСТІ І ФУНКЦІОНАЛЬНА АКТИВНІСТЬ ГЕБ ПРИ РИТМІЧНИХ ХОЛОДОВИХ ВПЛИВАХ

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Вивчені ультраструктури ГЕБ і синаптичного апарату гіпоталамусу щурів при гіпотермії. Досліджен секреція, рецепція і проникність ГЕБ для моноамінів. Показано, що ритмічні холодові впливи підвищують в 3–4 рази проникність ГЕБ для ^{3}H-норадреналну, але не для ^{3}H-серотоніну. Електронограми свідчать про те, що після холодових впливів синаптичний апарат перебуває в стані напруження, а в ендотелії мікросудин виявляються клатринові везикули, активуються процеси рецепторного трансцитозу. Нейротрансмітерні процеси, які обумовлюють проникність ГЕБ, можуть бути пусковим нейрофізіологічним механізмом зміни центральної терморегуляції при гіпотермії.

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К МЕХАНИЗМАМ РЕГУЛЯЦИИ ПРОНИЦАЕМОСТИ ГЕМАТОЭНЦЕФАЛИЧЕСКОГО БАРЬЕРА ОХЛАЖДЕННОГО МОЗГА. СООБЩЕНИЕ 4. УЛЬТРАСТРУКТУРНЫЕ ОСОБЕННОСТИ И ФУНКЦИОНАЛЬНАЯ АКТИВНОСТЬ ГЕМАТОЭНЦЕФАЛИЧЕСКОГО БАРЬЕРА ПРИ РИТМИЧЕСКИХ ХОЛОДОВЫХ ВОЗДЕЙСТВИЯХ

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Изучены ультраструктуры гематоэнцефалического барьера (ГЭБ) и синаптического аппарата гипоталамуса крыс при гипотермии. Исследованы секреция, рецепция и проницаемость ГЭБ для моноаминов. Показано, что ритмические холодовые воздействия увеличивают в 3–4 раза проницаемость ГЭБ для ^{3}H–норадреналина, но не для ^{3}H–серотонина. Электронограммы свидетельствуют о том, что после холодовых воздействий синаптический аппарат находится в состоянии напряжения, а в эндотелии микрососудов обнаруживаются клатриновые везикулы, активируются процессы рецепторного трансцитоза. Нейротрансмиттерные процессы, обусловливающие проницаемость ГЭБ, могут оказаться пусковым нейрофизиологическим механизмом изменения центральной терморегуляции при гипотермии.

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MEMBRANE STRUCTURES, DETERMINING PHENOTYPICAL CHARACTERISTICS AND FUNCTIONAL STATUS OF HEMOPOIETIC CELLS; THEIR POSSIBLE MODIFICATION UNDER THE ACTION OF CRYOPRESERVATION FACTORS. PART II

In continuation of the analysis of the state of the receptor structures on hemopoietic cells, IL-1 receptor, in particular, which the authors started earlier (rf.."Problemy kriobiologii", 1995, №3), it should be noted that formation of a IL-1+IL-1R complex may activate thyrosine kinase, trigger phosphorylation of the membrane proteins, and in some cases, during the action of IL-1 on the glycoproteid-treated cells of human peripheral blood, to bring about phosphorylation of the protein with a molecular mass of 65 kDa.

A cascade of biochemical events with the development of a corresponding effect, apparently, is not limited by phosphorylation of the protein structures. The data which support possible internalization of the ligand with its subsequent degradation are rew. Using electron microscopy and autoradiography the authors managed to demonstrate the development of a particular fraction of the internalized IL-1 in the nucleus [28]. A complexity of the nature and action of the IL-1 on the cells is also confirmed by the fact that IL-1 rapidly increases the exchange of protein kinase C in the *jurkat* cells, which are not expressed on the IL-1 membrane.

Availability of the stimulating effect with respect to the hemopoietic precursors after injection of IL-6 to the mice [14] makes it possible to assume on the probable presence of the receptor to IL-6 on these cells. The results of studying the action of IL-6 on the primitive stem cells, obtained following a corresponding treatment of the bone marrow of the mice with 5-fluorouracyl (5-FU) and a certain class of the monoclonal antibodies (MAB), are rather interesting. It was reported that for the population, which is resistant to 5-FU and growing in the presence of the optimum concentrations of CSF-1+IL-1+IL-3, the addition of IL-6 resulted in a dose-dependent rise in the amount and volume of the colonies. At the same time, addition of the IL-6 to the population, sensitive to 5-FU, and stimulated by CSF-1+IL-3, did not produce a similar effect. These results testify to the availability of

the subpopulations of hemopoietic cells in the bone marrow, which possess high proliferative potentials (HPP-CFU), which are realized in the presence of the IL-6, thus supporting the synergy of action of the IL-6 with other growth factors with regards to the stem cells. The data on the addition of the IL-6 to increase the content of CFU_c in the long-term bone marrow culture may testify to the manifestation of the stimulating activity of the IL-6 at late stages of hemopoiesis [21]. Unfortunately, it is impossible to find out whether a direct or mediated action of the IL-6 is due to the CSF induction by the accessory cells. It is known, for example, that IL-6 acts as an additional signal in a mitogen-dependent T-cell activation, and the activated T-cells may inject growth factors into the medium. The results of the investigations of the leukosic cells, which are a homogeneous population, make it possible to assume that at least some of the signals may be of the direct action, though it should be taken into account that the leukosic cells in themselves do not infrequently secrete GM-CSF, G-CSF, IL-1. Several years ago experimentation with mice showed a direct action of IL-6 ($IF\beta_2$) on the colony-forming activity of the CFU-GM, and its ability to maintain the colony-formation of the early blast-forming cells was reported [22].

Expansion of the field of application of hormones of the adrenal gland in clinical practice and good evidence of their diversified action on the cells, organs and systems of the human organism prompt the urgent necessity to define a possible acceptance of these powerful bioregulators by the hemopoietic precursors, i.e. the availability of the receptors to glucorticoids (GC) on them. Unfortunately, there is no direct convincing evidence of the availability of the receptors to GC on the membranes of the stem hemopoietic cells, though some researchers do speculate on the problem of the involvement of GC in the modulation of the cytokine receptors on the cells of the lymphohemopoietic system. It was reported, however, that injection of IL-1 to the mice resulted in a temporary inhibition of binding of G-, GM-CSF, FNO with corresponding receptors of the cells of bone marrow. Dexametazone induced a rise in binding of the IL-1 with the cells of the lymphohemopoietic complex in the *in vivo* system, and, vice versa, adrenalectomy reduced binding of the IL-1. It should be reported that the results of these studies are not only of theoretical, but of practical importance as well, testifying to the necessity of introducing the corresponding corrections into a composite therapy, involving application of glucocorticoids during transplantation of the bone marrow, cryopreserved, in particular, in which a dysbalance of the regulatory-acceptor cells is possible, and, hence, of the cytokin production [32], and a change in the structure-functional organization of the receptor apparatus of the cell [5]. The receptors to GC are rather labile systems, which alter their functional activity after changing the temperature, ionic strength of the solution; they are sensitive to the action of antagonists of calmodulin-vinblastine, propanediol [9]. A major role of the intracellular Ca^{2+} and Mg^{2+} in the processes of transformation of a receptor-glucocorticoid complex should be mentioned [20]. The data on the state of a "hormone-receptor" complex following its interaction with chromatin are also important. On the basis of the data available on the fact that such an interaction results in the formation of a new conformation structure, which provides for a rise in the synthesis of mRNA, the authors hypothesize that these newly formed structures may exist in the active state for a long time. A receptor protein is thermally labile, and contains sulfhydril groups. Oxidation or alkylation destroys a binding activity of the receptor. It cannot be excluded that such a change in the conformation of the solution may occur after cryopreservation, since the barrier properties of the membranes are disrupted due to the temperature-dependent phase-structural transitions of lipids and intensification of their peroxidation, there occurs a leakage of the transported Ca^{2+} out of the SPR both via a system of the active transport, and

by means of the passive diffusion via transmembrane defects. The impairments of the barrier properties of the membrane result in the elimination of various proteins and enzymes out of the cytoplasm [2]. Even under physiological conditions the receptor may be returned to the cytoplasm and expand the pool of the newly synthesized receptors, or it may degrade. Still, if we assume the availability of the receptors to glycocorticoids on the hemopoietic cells while developing proper modes of applying glycocorticoids during bone marrow transplantation, then, apparently, certain peculiarities of expression of these structures on other cells as well should be taken into consideration. For example, on the mononucleated cells of human peripheral blood the level of these receptors is going down with age, while seasonal changes do not affect this feature, and in women the amount of these receptors is somewhat lower, the circade variants of cortisole in plasma do not alter the level of the receptors on the membrane, etc. It is also important that the number of these receptors to glycorticoids may be reduced during their long-term application [31].

In a wide range of the receptor structures of hemopoietic cells the important part is played by the receptors to retinoic acid. The latter significantly affects proliferation and differentiation of many hemopoietic cells. The mechanism of this action is still unclear. Recently several receptors of the retinoic acid were cloned [24]. When the results of these investigations were summarized, it was concluded on the expression of the retinoic acid to be independent either of the level of differentiation of hemopoietic cells or on the cell cycle. This receptor had a short life period and was easily produced under the action of the inhibitors of the protein synthesis. The myeloid precursors were found to be resistant to the action of the retinoic acid during the congenital agranulocytosis of the bone marrow (the stimulation of proliferation is missing), thus supporting an important role of the retinoic acid in providing for the myeloid growth of hemopoiesis.

Recently a lot more attention is paid to the product of a *c-kit* gene – transmembrane tyrosine kinase receptor for the new hemopoietic factor, which is called a growth factor of mast cells, *c-kit*-ligand or a stem cell factor, stimulating proliferation of the hemopoietic precursors, which is expressed in high concentrations on the hemopoietic cells, brain tissue and placenta [34]. The expression of the product of a *c-kit*-gene was shown to be modulated by the hemopoietic growth factors, including IL-3, GM-CSF and erythropoietin. These growth factors reduce the level of a *c-kit*-protein and its mRNA. Apparently, this is one of the links of realization of the mechanism, providing for a balanced state of proliferation and differentiation of the key elements in the hemopoietic system. With the help of MAB to the *c-kit*-product, CD33, CD71 structures of hemopoietic precursors of various extent of maturation and using a method of a flow cytofluorometry the authors investigated the distribution of these molecules on the hemopoietic cells. Ten samples of the normal human bone marrow exhibit some 19–51% of $CD34^+$ cells, which express *c-kit*-receptor. A considerable part of these cells co-expressed CD33 (52±23%) and/or CD71 (62±26%), which are the markers of the committed colony-forming cells [29]. This fact seems very interesting, since it testifies to a possible regulation of the process of proliferation not only in the terminal, but in the stem department of hemopoietic precursors as well.

Admission of the concept of the interregulation of the CNS, endocrine and immune systems requires a more detailed study of the action of the products of the CNS activity on the cell of the lymphohemopoietic complex. The authors gained strong evidence in favor of the involvement of the endogenous opioid peptides in the regulation of the vital functions under physiological and extreme conditions. Many researchers investigated the action of opioids on the bone marrow hemopoiesis. Assumption on the participation of the opioids in the hemopoiesis is based on the identification of a CALLA-marker on the immature hemopoietic cells with the help

of enkephalinase [15]. The clonal cultures of the human and murine bone marrow stem cells, isolated with a cell sorter, were incubated with leu- and methionine-enkephalin in the concentration of 10^{-6}–10^{-15} M in the presence or absence of the specific opioid antagonist – naloxon. Both enkephalins reduced the number of CFU-GM and clusters by 50–85% as compared with control samples. Naloxon eliminated the effect of opioids. A synthetic analogue of leu-enkephalin – dalargin – also exercises an inhibiting action on the erythro- and granulopoiesis; and this action is realized not only during the balanced hemopoiesis, but under the influence of the postmobilization stress as well. A combined injection of the preparations did not result in the hyperplasia of the bone marrow, which is characteristic of the adaptation syndrome, since naloxon eliminated a stimulating effect of the endogenous meth-enkephalin on the hemopoiesis during immobilization stress. Such an effect of the antagonist of the opioid receptors, as described elsewhere [4], may be related to the diversified action of enkephalins on the hemopoiesis during stress. For instance, injection of meth-enkephalin to the mice, exposed to immobilization, exercises a stimulating action on hemopoiesis. Under stress conditions the adrenal glands produce mainly meth-enkephalin, the action of which, apparently, may result in hyperplasia.

The data are available on the effect of opioids on the growth of colonies of a granulocyto-macrophagal series in the *in vivo* culture of the bone marrow of healthy adults in the presence or absence of growth factors. Leu- and meth-enkephalins stimulated growth of the bone marrow only provided their own growth was poor. During enhanced cell growth leu-enkephalin inhibited proliferation of cells, while meth-enkephalin did not affect it. When using a stimulating growth factor both enkephalins either did not affect, or inhibited proliferation of the bone marrow cells. The data submitted demonstrate that, in the first place, opioids are also involved in the maintenance of the balanced hemopoiesis in the organism, and, in the second place, they are capable, apparently, of affecting both early and late precursors of hemopoiesis. Though one cannot exclude both direct (via opioid receptors on the hemopoietic cells) and mediated (via receptors on the cells of microenvironment) action of enkephalins on hemopoiesis.

A study of physical-chemical and immunologic properties of the receptors of the opioid peptides in the brain and leukocytes of the peripheral blood showed that the receptors of the opioid peptide is functionally bound with cAMP, and, apparently, its activity modulates Ca^{2+} and K^{+} channels. The receptors possess similar biochemical and functional properties [19].

Among the works on the interaction of the nervous and lymphohemopoietic systems some reports deal with the effect of the mediators of adrenergic and cholinergic nervous terminals on the proliferation and differentiation of the hemopoietic cells. The interaction of specific antagonists with β-adrenergic, nicotine-cholinergic and histamine-H_2 receptors may alter the state of the cell cycle of the bone marrow stem cells, triggering CFUs into the *S*-phase of the cell cycle [16].

Some researchers [16] investigated the concentration of the adrenergic and histamine receptors on the cells of the bone marrow, fractionated in a Ficoll gradient, and studied binding of various ligands with these receptors. The authors reported on a 2-3-fold increase in the density of β-adrenoreceptors and H_2 histamine receptors on the cells of the fraction, which contained a major part of the early progenitors of the hemopoietic cells. There was also demonstrated the availability of certain receptors to agonists on the bone marrow hemopoietic cells. Proliferation of CFUs is altered under the action of the agonists of the muscarin-cholinergic receptors [25]. The ligands of β-adrenergic receptors enhance growth of the committed precursors of erythropoiesis and granulocytes [12]. The role of

the given receptors in the manifestation of the functional activity of cells still remains vague. It was reported though that endogenous histamine of the bone marrow may trigger CFUs into the *S*-phase via H_2-receptors [16]. Activation of phosphodiesterase by imidasole suppresses such a triggering of CFUs in response to the action of H-methyl-histamine, testifying to the involvement of the cyclase system in the response, induced via the H_2-receptors of histamine. On the other hand, inhibition of phosphodiesterase represents a possible way of triggering CFUs into the *S*-phase, which may result in the self-renewal of CFUs or enhancement of production of the committed precursors. While a possible presence of the receptors to histamine on the hemopoietic precursors of various levels of differentiation and their activation during formation of the ligand-receptor block cannot be excluded, it should be noted that activation of the colony-formation by endogenous histamine may be mediated by the activation of the contrasuppressive cells of the bone marrow, thus eliminating antimitogenic block [3]. With respect to the above it is worth mentioning that one of the reasons for the earlier reported reduction in the proliferative activity of the bone marrow stem cells after cryopreservation may be not only a change in the structure-conformational parameters of the receptor cell apparatus, but the impairment in the functional properties of the adenylate cyclase complex as well, which is manifested in the first place in the reduced enzymatic activity in the presence of catecholamines, isoproterenol and fluoride ions [2]. It was found that freeze-thawing may be followed by the impairments either in the structure of the active centers of β-adrenoreceptors, or the processes of signal transmission in the cyclase system.

The problem of the activation of phosphatidyl esterase and the effect of this enzyme on the functional activity of hemopoietic precursors is of special importance. It was reported elsewhere [5] that the behavior of hemopoietic cells (CFUs and CFUc) after cryopreservation differ in principle from that in the physiological state, which is manifested by changes in the proliferative activity, which is believed to be a consequence of the impairments in the metabolic activity, the state of the receptor apparatus, development of the nonlethal injuries. Apparently, an additional factor (but surely not the only one) is a change in the activity of phosphodiesterase. Inhibition in CFUs by phosphodiesterase (PDE) results in triggering of the DNA synthesis, which testifies to the ability of CFUs to synthesize cyclic nucleotides, and it may maintain the amount of cAMP at a subtriggering level in the hemopoietic cells, being in the state of a relative relaxation [16]. In this respect a rise in the level of PDE brings about a manifested blockade of the mechanism of transfer of CFUs in the state of proliferation. At the same time freeze-thawing is a factor, which causes a manifested rise in the activity of this enzyme [2], which follows from it being influenced by proteases, outflowing from the lysosomes, injured in the process of cryopreservation [8]. Although in the cells, which are the "candidates" into stem hemopoietic elements, the content of the lysosomes is reduced significantly [6], this link of activation of PDE in hemopoietic cells cannot be neglected during cryopreservation.

Apparently, yet another evidence of the interaction of a lymphogenetic complex with the CNS is community of features in the expression of one of the membrane structures – Thy-1 antigen. This antigen was shown to be related mainly with the grey substance of the brain in different human and animal species, and its synthesis is realized by neurons.

The results of investigations, conducted in recent years, allow to assume on the availability of a Thy-1 antigen on the hemopoietic cells of the murine bone marrow. Some authors believe that among a Thy-1 population of the bone marrow cells there are CFUs, aged 8 and 12 days [5]. It was shown that the chemical structure of a Thy-1 antigen is that of a glycoprotein [1]. A carbohydrate composition

of a Thy-1 antigen in mammals of different species is also different. Attention is surely to be paid to the assumption that a carbohydrate component is common to a Thy-1 antigen and monosialoganglioside, and the latter was thought for some time to be a Thy-1 antigen. The differences in glycosylation of the polypeptide chain of this antigen in the tissues of the thymus and brain may testify to a different function of a Thy-1 antigen in these organs. There is evidence on the involvement of a carbohydrate component of a Thy-1 antigen in the functioning of T-lymphocytes. Elimination of sialic acid from T-lymphocytes by means of treatment with neuraminidase results in the loss of the lymphocyte ability for homing in the corresponding organs. A Thy-1 antigen was shown to mediate the adhesion of the murine thymocyte to thymic epithelial cells via a Ca^{2+}-dependent mechanism. The data are available on the action of a Thy-1 antigen on the immune genesis. A Thy-1 antigen is thought to be involved into the process of activation of T-lymphocytes with participation of other cells, macrophages, in particular. Apparently, activation of T-lymphocytes via a Thy-1 antigen is realized during the interaction of this antigen with other membrane components of the cell, such as antigens of histocompatibility – h-2, Fc-receptor [26]. One cannot exclude the interaction of a Thy-1 antigen with a glycolipid of the ganglioside nature. There was found a relation between a Thy-1 antigen and the membrane phospholipid (phosphatidyl inosite-4, 5-biphosphate) (PTI), the hydrolysis of which under the action of phospholipase C is known to result in the activation of the immunocompetent cells [23]. Hydrolysis of phospholipids due to the activation of the intramembrane phospholipases after cryopreservation is to be accompanied, as judged from the above, by the activation of the immunocompetent cells (ICC), however, this is not supported by the experimental investigations. It is speculated that the activation of ICC is exercised due to the action of several activation signals [11]: primary antigen-specific, which transfers the cell into a G_1 phase, and the secondary one which is immunologically nonspecific, and which is realized by various lymphokines, being required for the transfer of the cell into the S-phase. It is probable that after the first signal is supplied the cryopreserved cells (even after a temporary shedding of the structures, which accept it) are capable of activation, however, the absence of the second signal (death, impairment of the ability of the regulatory cells to produce cytokines after cryopreservation) results in the observed reduction in the extent of the proliferative response of the cryopreserved ICC.

Currently it is still doubtful whether the effect of a rise in the activity of the hemopoietic cells occurs, alike ICC, due to the activation of the inositol-phosphate cycle, though the occurrence of these events is probable, since the existence of a covalent bond of the protein part of the receptor with PTI, beside Thy-1, has been confirmed for many proteins. An example of the covalent-bound receptors on hemopoietic cells are the structures Ly6, LFA [7]. The interaction of the PTI-bound receptor with the ligand results in the activation of phospholipase C, which eliminates binding of the membrane proteins with PTI. Here not all the protein-glycolipid complexes are fused with similar rates. A Thy-1 antigen is relatively stable, while the antigens Ly6 and LFA are rapidly transformed into the soluble forms. Release of the PTI-bound proteins during activation of cells is of great importance in the processes of adhesion, homing of cells, transition of the intracellular stimuli and it depends, in the first place, on the exchange of the membrane phospholipids and enzymatic operation of phospholipases. It is very difficult to follow the functioning of the mechanism of the intracellular activation of stem hemopoietic cells in response to the action of the regulatory signals due to widely known difficulties in collecting a homogeneous population of hemopoietic cells. However, with some admission this job may be realized using lymphocytes as an example. It was shown that the early transformation of lymphocytes during

mitogenic stimulation is accompanied by the activation of the PTI-cascade with the involvement of phospholipase C, activation of protein kinase C, the enzyme, which is responsible for the phosphorylation of the specific proteins, playing key roles in the genome activation during transformation of lymphocytes. Activation of cell also relates to the changes in the fraction of phosphatidyl choline, the content of which increases in the membrane outer surface, reducing the viscosity of the layer and increasing income of Ca^{2+} into the cell, which, as combined with methylation of phosphatidyl choline, is accompanied by the activation of phospholipase A_2 and release of arachidonic acid from phosphatidyl choline with its subsequent transformation and accumulation of the local tissue regulators – prostaglandins [11].

A system of metabolism of phosphatidyl inosides, together with the adenylate cyclase and ion-transporting systems, is the basic system of regulation of physicochemical processes in the membranes and cell as whole when responding to various stimuli, including temperature and osmotic signals [2]. For example, a reduction in the fluidity of the membrane at 4 °C increases a possibility of the low avidic interaction of the suppressor factor, produced by the regulatory cells of the bone marrow, with the corresponding receptors of the P-815 mastocytoma cells [10]. At present the action of this factor on the hemopoietic precursors has been verified, hence, some aspects of the problem of a temperature-dependent variability of the functional status of these cells may be interpreted differently. At the same, a cross-binding of the murine IL-3 and GM-CSF with the receptor is missing at 4 °C, though at 37 °C IL-3 reduced the binding with GM-CSF [33].

All the data available on the action of cryopreservation on the state of the antigen-receptor structures relate, mainly, to investigating the characteristics of cryopreserved lymphoid cells. It should be noted that the results of these investigations are rather contradictory. For example, it was shown that during cryopreservation of lymphocytes of the human peripheral blood some functional properties of these cells are changed, but the expression of the surfacial antigenic structures did not change [17]. Other researchers [27] report that an insignificant reduction in the total amount of T-cells, isolated by the method of a spontaneous rosette-formation with ram RBC, was followed by a considerable decrease in the amount of $T\mu$ and $T\gamma$ cells, which testifies to a higher cryosensitivity of Fc-receptors as compared with E-receptors. Cryopreserved polymorphonucleated leukocytes of the peripheral blood lost the ability for binding the opsonized IgG and C_3 components of the complement [30]. Similar results were also obtained after the cells were exposed to osmotic stress in the hypertonic medium, which models a freeze-thawing cycle. The said membrane structures were identified in the cell supernatant, which enabled the authors to assume that after cryopreservation there occurs shedding of the membrane structures. Of the similar importance are the results of the works, which report on the change in the expression of the antigens of the bone marrow cells during some types of leukemia due to cryopreservation according to a 2-stage program [18]. The highest cryosensitivity was characteristic of the stronger differentiated leukosic cells. Different sensitivity to cryopreservation of various receptors of one and the same cells was verified in the experiments [13] with cryopreserved bone marrow when analyzing the state of CFU-GM: following cryopreservation these cells lost sensitivity to a colony-stimulating activity of the conditioning medium (GCT), while maintaining sensitivity to the factors of the feeder layer.

Conformational rearrangements of the glycoprotein complexes, protein-lipid components, shedding, stipulated by changes in *pH* of the medium, concentration of salts, temperature, nucleation during cryopreservation, may underlie a reduction in the ability of the $Thy\text{-}1^+$ cells of the bone marrow, including hemopoietic cells – CFUs, to attach to a MAB-antigen of Thy-1.2 [5]. Besides, a temporary

inhibition of the metabolic activity of hemopoietic cells after cryopreservation was verified. For example, CFUs, modulated by a Thy-1.2 antigen ("withdrawal" of a Thy-1.2 antigen by forming a complex antigen+antibody+antibody), demonstrated a manifested slowing down of the rate of synthesis of the said structures as compared with the dynamics of the similar processes in the native antigen-modulated CFUs [5]. Hence, judging from the above changes in the state of Thy-1.2 of the membrane structure it may be concluded on the cryopreservation to apparently result both in the changes of the state of the antigen-receptor structures of hemopoietic cells, and in a decrease in their generation due to inhibition of the metabolic processes. On the whole this results in the impairments of the intercellular interaction, and finally, of the functional status of hemopoietic cells.

Application of molecular-genetic, immunologic methods of investigation yielded the data on the submolecular structure of some receptor molecules of hemopoietic cells and on the functional role of their separate domains. However, the problem on the paths of regulation of the interrelations between the receptor and various components of the cell is very complicated. It remains unclear to which extent various mechanism of the signal transmission are stipulated in every particular case by the receptors of various types, and how many receptors are there all in all.

Thus, a conducted survey of the literature reports testify to the availability of the membrane structures on hemopoietic cells, which realize a wide range of functions. Most of them are sufficiently labile, and, probably, most "vulnerable" to the action of unfavorable factors of the environment, cryopreservation including. Application of cryopreserved bone marrow in experimental and clinical practice and a verified phenomenon of the impairments of the myelotransplant functioning in the recipient organism determine the necessity to newly evaluate the state of the cryopreserved hemopoietic tissue and develop the methods of cryopreservation for this material. That is why one of the criteria of the efficiency of one or the other method of cryopreservation of hemopoietic cells is provision for a functionally active state of their membrane-receptor structures. Evaluation of survival of the membrane-receptor structures should be included into the evaluation of the morphofunctional state of hemopoietic cells as a whole. The data on the changes in the expression of antigens and functional activity of the receptor structures of hemopoietic cells after cryopreservation will make it possible to establish new mechanisms of the protective action of the bone marrow being transplanted. Considering a possible modification of the antigen-receptor spectrum of the membranes of hemopoietic cells after cryopreservation and the ability of the humoral substances to enhance the expression of the surfacial structures, application of the inductors-stimulators of the expression of receptors to the regulatory hemopoietic factors with the aim of increasing the efficiency of utilization of the cryopreserved bone marrow becomes very promising.

ABBREVIATIONS:

GM(G)-CSF – granulocyto-macrophagal (G – granulocytic) colony-stimulating factor
GC – glycocorticoids
ICC – immunocompetent cells
IL – interleukin
IL-1P – receptor to IL-1
IF – interferone
CFU-GM – colony-forming units, which form colonies, comprising granulocyto-macrophagal cells
CFUc(s) – colony-forming unit in the culture (spleen)
SPR – sarcoplasmic reticulum
PDE – phosphodiesterase
FTN – factor of tumor necrosis
5-FU – 5-fluorouracyl

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МЕМБРАННІ СТРУКТУРИ, ЯКІ ВИЗНАЧАЮТЬ ФЕНОТИПІЧНІ ХАРАКТЕРИСТИКИ І ФУНКЦІОНАЛЬНИЙ СТАТУС КРОВОТВОРНИХ КЛІТИН; МОЖЛИВА МОДИФІКАЦІЯ ПІД ДІЄЮ ФАКТОРІВ КРІОКОНСЕРВУВАННЯ. ЧАСТИНА II

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Продовжена характеристика ряду поверхневих структур кровотворних клітин кісткового мозку та розглянута їх участь в рецепції регуляторних сигналів, які забезпечують гемопоез. Наведені дані, які свідчать про зміну функціональної активності гемопоетичних клітин в результаті порушення (модифікації) стану антигенних структур після кріоконсервування.

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МЕМБРАННЫЕ СТРУКТУРЫ, ОПРЕДЕЛЯЮЩИЕ ФЕНОТИПИЧЕСКИЕ ХАРАКТЕРИСТИКИ И ФУНКЦИОНАЛЬНЫЙ СТАТУС КРОВЕТВОРНЫХ КЛЕТОК; ВОЗМОЖНАЯ МОДИФИКАЦИЯ ПОД ДЕЙСТВИЕМ ФАКТОРОВ КРИОКОНСЕРВИРОВАНИЯ. ЧАСТЬ II

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Продолжена характеристика ряда поверхностных структур кроветворных клеток костного мозга и рассмотрено их участие в рецепции регуляторных сигналов, обеспечивающих гемопоэз. Приведены данные, свидетельствующие об изменении функциональной активности гемопоэтических клеток в результате нарушения (модификации) состояния антигенных структур после криоконсервирования.

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ALTERATIONS IN PROTEIN SPECTRUM OF A *TENEBRIO MOLITOR* LARVAE DURING COLD ACCLIMATION

Protein spectrum of the tissues of *Tenebrio molitor* (family of *Tenebrionidae*) larvae, which avoid freezing, was studied under various temperature modes. A method of SDS-electrophoresis in polyacryl amide gel (PAAG) showed that during low temperature acclimation of larvae a possibility of the appearance of a 65 kDa protein significantly increases as compared with that in the control samples, kept at room temperature. A considerable reduction in the content of the given protein in the samples, which prior to acclimation were injected with the inhibitor of translation – cycloheximide, supports a possibility of synthesis of this protein during cold acclimation *de novo*.

Currently one of the urgent problems of cryobiology is the study of the mechanisms of the adaptation responses of animals to cooling. It was reported elsewhere [6] that during cold acclimation the proteins with molecular masses of about 9 kDa are accumulated in the organisms of some animals, insects, in particular, which avoid freezing. These proteins facilitate supercooling of the extracellular fluids of the body down to –9÷–20 °C [2–5], depending on the type and conditions of cold acclimation. Besides, some enzymatic systems are also activated, which are responsible for the synthesis of the low molecular antifreezers of the non-protein

nature [2–5, 7, 8]. The data submitted testify to a programmable alteration of biosynthetic processes in the cells, thus facilitating cold acclimation of animals; in general the newly synthesized proteins may be designed as proteins-adaptogenes. It cannot be excluded that beside the above low molecular proteins, the synthesis of which is activated on cold, the period of acclimation witnesses activation of synthesis of other proteins-adaptogenes, in particular, shaperon proteins, etc. [5]. With the aim of identifying the spectrum of proteins, which are synthesized during cold acclimation, the authors performed a study of the quantitative and qualitative spectrum of proteins out of the tissues of the larvae *Tenebrio molitor* (fam.*Tenebrionidae*), which avoid freezing.

The larvae *Tenebrio molitor* (fam. *Tenebrionidae*) out of the laboratory population were used in the experiments. The average weights of the larvae were 100–150 g. The larvae were acclimated at 2–4, 7–10, 12–15 °C in the refrigerator. Some larvae were acclimated under the conditions of a prolonged photoperiod (18-hour light:6-hour darkness). The inhibitor of translation – cycloheximide – was injected to the larvae at a dose of 3 μg/species using a Hamilton syringe. The concentration of the cycloheximide solution was 1 μm/1 μl. Every larva was homogenized in a glass-made homogenizer in 400 μl 0.1 M phosphate buffer, *pH* 8, to which an alcohol solution of phenyl methylsulphonyl fluoride (PMSP) – the inhibitor of proteases – was added until a finite concentration of 0.3 μg/ml for inactivating proteases. A 96%-ethanol solution was added to the aliquote of the homogenate in a 1:1 ratio [6]. Extraction was conducted with intensive stirring for 25–30 min. Then the samples were centrifuged (3000 rpm for 5 min). The supernatant (alcohol extracts) were collected for subsequent experimentation. With the aim of preparing the samples for DCH-electrophoresis 50 μl of the supernatant were mixed with 50 μl of the buffer for dissolving the samples (0.05 M *tris*-HCl, *pH* 6.8; 20%-glycerol; 0.003 M trylon B; 2%-dodecyl sodium sulphate; 0.4 mg/ml sodium aside; 0.1 mg/ml PMSP; 5%-mercaptopropanediol; 0.01% bromphenol blue), and boiled for 3 min on water bath. In some experiments the samples were prepared from the mixture of the alcohol extracts, collected from 5–6 larvae. The samples were stored in a freezer at –10 °C. A DCH-electrophoresis was performed in a polyacryl amide gradient gel (12.5–25%) according to a routine technique [1]. Fixation and staining of proteins in the gel was conducted as described elsewhere [1].

Fig. 1 shows the data on a DCH-electrophoresis in PAAG of proteins of the alcohol extracts, isolated from the tissues of the larvae *T.molitor*. It follows from Fig. 1 (the samples 1 and 2 demonstrate the protein spectra of the acclimated and non-acclimated larvae, respectively) that after cold acclimation, lasting for 14 days and longer, there occurs a change in the qualitative composition of the proteins; in particular, a pronounced appearance of the band with a molecular mass of 65 kDa is observed. Attention is to be paid to the changes in the fractions 30 kDa, 16–18 kDa and 5–10 kDa. It is evident that some components of these fractions, which are available in larvae, kept under acclimation conditions, are missing in control larvae, in particular, those of 30 and 5–10 kDa;

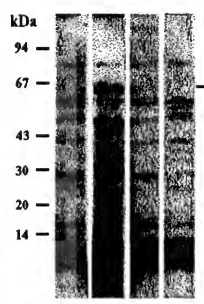

Fig. 1. Electrophoregram of the alcohol-soluble proteins out of the tissues of the non-acclimated larvae *Tenebrio molitor* (1), acclimated larvae (2), deacclimated larvae (3), larvae acclimated in the presence of cycloheximide *in vitro* (4). M – markers of molecular mass

however, a group of proteins of 16–18 kDa in the acclimated larvae is reduced considerably. There are also certain differences in the range of 80 kDa, however, the authors failed to acknowledge whether there exists a dependence between the temperature, at which the insects are kept, and the nature of changes in this area. Apparently, these differences relate to the individual intrapopulation protein polymorphism. The analysis of the reports available showed that the data on the molecular masses, determined by a DCH-electrophoretic method (i.e under denaturating conditions), on the ethanol-soluble proteins and peptides, which appear during acclimation, are missing at the moment. As reported elsewhere [6] at least one of the components of the alcohol extract out of the tissues of the cold-acclimated larvae *T.molitor*, which possesses thermohysteresis activity, is of the molecular mass of 9 kDa, which was determined by a method of gel-filtration, i.e. under conditions, which provide for the maintenance of the native conformation of peptides. Due to this it seems difficult to compare the authors' results with the results obtained by other researchers when studying antifreeze-proteins.

It should be noted that the protein with a 65 kDa protein is present in some species at room temperature (approximately in 25% of species out of the studied selection). Following acclimation the amount of species, which contain this protein, increases up to 75%. Upon return of the insects to room temperature (the authors called this process "deacclimation"), the protein spectrum starts to change, and by the 7th day the protein samples of the deacclimated larvae become similar to those of the non-acclimated larvae (sample 3 in Fig. 1; approximately in 25% of the deacclimated samples the protein 65 kDa is identified).

It is worth mentioning that the qualitative changes in the protein spectrum, at least those in the band of 56 kDa, are related, apparently, to the protein synthesis *de novo*, since its appearance is blocked upon injection of the translation inhibitor – cycloheximide – into the organism (sample 4 in Fig. 1). The densitometric data testify to the fact that a reduction in the content of the given protein in the larvae, injected with cycloheximide, was 50% as compared with the content of this protein in the acclimated larvae, which were not injected with the inhibitor of the protein synthesis (relative content of a 65 kDa protein was 32.4 and 16.3% for the acclimated larvae without injection of cycloheximide and the larvae, acclimated after cycloheximide administration, respectively). It should be noted that injection of cycloheximide results in a minor reduction of the protein content in the obtained samples on the whole. Only 32% of the acclimated larvae, injected with cycloheximide, demonstrated appearance of a 65 kDa protein.

Elsewhere [8] it was reported that initiation of the synthesis of the antifreeze proteins occurs under the influence of a short-term photoperiod (10–11-hour light: 13–14-hour darkness). There is the evidence of the fact that antifreeze proteins are synthesized in the larvae, which are kept in darkness and exposed to a certain temperature (8 hours at 25 °C:16 hours at 17 °C). In this respect the authors studied the action of the prolongation of the photoperiod and illumination on the appearance of a 65 kDa protein during low temperature acclimation. Some larvae were acclimated during a long-term photoperiod (18-hour light:6-hour darkness), and these larvae also demonstrate the appearance of a 65 kDa band. Besides, some larvae were deacclimated in the darkness. A 65 kDa protein was missing in the protein patterns of the larvae, deacclimated in the darkness. The data obtained make it possible to conclude that the reason for the appearance of a 65 kDa protein is just the lowering of the temperature. Apparently, this protein belongs to the group of the cold proteins-adaptogenes, which differ from the antifreeze proteins and the regulation of the synthesis of which is realized by other means.

Thus, during low temperature acclimation (with the prolongation of more than 7 days) there become evident quantitative and qualitative changes in the

protein spectrum in the acclimated larvae *Tenebrio molitor*, which is supported by the data of a DCH-electrophoresis in PAAG. It was found that during acclimation under the lowered temperature the new protein band appears with a high frequency, which corresponds to the protein with the molecular mass of 65 kDa. This band is missing in most species of the population at room temperature. A probable synthesis of this protein *de novo* is supported by a significant qualitative reduction of the given band in the protein patterns of the species, which were injected with cycloheximide prior to acclimation.

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ЗМІНИ У БІЛКОВОМУ СПЕКТРІ ЛИЧИНОК *TENEBRIO MOLITOR*, У ПЕРІОД ХОЛОДОВОЇ АККЛІМАЦІЇ

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Проведено дослідження спектру білків з тканин уникаючих замерзання личинок *Tenebrio molitor* (род. *Tenebrionidae*) в умовах різних температурних режимів. Методом ДСН-електрофорезу у ПААГі було встановлено, що при низькотемпературній акклімації личинок вірогідність появи протеїну 65 кДа значно зростає у контрольних об'єктах, які утримувались в умовах кімнатної температури. Суттєва редукція вмісту даного білка у об'єктів, яким напередодні акклімації був введений інгібітор трансляції – циклогексимід, вказує на можливість синтезу цього білка в умовах холодової акклімації *de novo*.

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ИЗМЕНЕНИЯ В СПЕКТРЕ БЕЛКОВ ЛИЧИНОК БОЛЬШОГО МУЧНОГО ХРУЩАКА *TENEBRIO MOLITOR* В ПЕРИОД ХОЛОДОВОЙ АККЛИМАЦИИ

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Проведено исследование спектра белков из тканей избегающих замерзания личинок большого мучного хрущака *Tenebrio molitor* (сем. *Tenebrionidae*) в условиях различных температурных режимов. Методом ДСН-электрофореза в полиакриламидном геле (ПААГ) было установлено, что при низкотемпературной акклимации личинок вероятность появления протеина 65 кДа значительно возрастает по сравнению с таковой у контрольных особей, содержащихся в условиях комнатной температуры. Существенная редукция содержания данного белка у особей, которым накануне акклимации был введен ингибитор трансляции – циклогексимид, указывает на возможность синтеза этого белка в условиях холодовой акклимации *de novo*.

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FUNCTIONAL STATE OF NATIVE AND CRYOPRESERVED ALLOHEPATOCYTES, DESTINED FOR CLINICAL APPLICATION

The authors studied the structural state, respiratory and protein-synthesizing activities, capture of xenobiotic biphenyl by native and cryopreserved hepatocytes of human fetal and cadaver livers, destined for clinical application. There were developed the methods of cryopreservation of hepatocytes in the medium with low concentrations of cryoprotectants without reducing their survival rate.

One of the promising trends in the up-to-date medicine is application of biological substrata of the the alloliver, isolated cells including, for treatment of the endotoxicoses of various etiology [2, 7]. A limited application of this method in clinical practice is due to the absence of the simple inexpensive methods of isolating viable cells, and of the adequate methods of evaluation of the viability at the stage of collection and long-term storage. The aim of the work was to study the structure-functional properties of hepatocytes following their isolation out the fetal human and cadaveric livers and cryopreservation.

For collecting hepatocytes use was made of human cadaveric liver, granted by the N.V.Sklifosovsky Scientific-Research Institute of Intense Care (Moscow, Russia). The period of the organ ischemia did not exceed 1.5–2.0 hours. Contraindications to collection were the information of the intense care medical service on the injury or illness of the organ, and the absence of the corresponding note of the forensic medical expert. The cells were harvested out of the donor material with the absence of the pathologic processes (cirrhosis, hepatitis, fat dystrophy) using the method, described elsewhere [10]. Hepatocytes out of the human fetuses of the later terms of development (16–26 weeks) were collected as described elsewhere [4] in the course of artificial birth deliveries by medical indications in the Department of the Pregnancy Pathologies of the 1st Municipal Hospital of Kharkov. The contraindications to collection were supplied by the anamnesis of the pregnancy. With the aim of the long-term storage of hepatocytes the subzero temperatures (–196 °C) were used. Freezing was performed with the use of two modes: a two-stage one, using slow rate of freezing (1 °C/min) down to –60 °C, followed by a temperature stop of 3–5 min, and subsequent immersion into liquid nitrogen (mode 1). The second mode involved only one-stage freezing at a rate of 1600 °C/min (mode 2). Freezing according to mode 1 was realized with a UOP-6 programmable freezer, and according tc mode 2 – with a method, described elsewhere [11]. With the aim of cryoprotection of hepatocytes in the process of freezing use was made of 3%- or 10%-solutions of dimexide (DMSO) and glycerol. The viability of hepatocytes was evaluated by trypan blue staining. The number of cells was counted in a Goryayev's chamber. Histologic studies were conducted as described in [6], and the cell fine structures were investigated as described elsewhere [9].

The level of endogenous respiration and stimulation by the increasing (1 mM, 10 mM) concentrations of succinate was evaluated in the medium, containing (mM): KCL – 5.0; dithiotretoil – 2.0; KH_2PO_4 – 0.4; $NaHPO_4$ – 0.4; EDTA – 2.0; sucrose – 250.0; albumin – 1% using a Clark electrode with a PA "Prague" polarograph. The cell concentration in 1 ml was $2x10^6$ on the average. A protein-synthesizing ability of hepatocytes was determined by incorporation of labeled amino acids into total proteins [5]. Capture of xenobiotic by hepatocytes was measured according to the fluorescence intensity with a Hitachi MPF-2A

spectrofluorimeter [12] after 20-min incubation (37 °C) of the cell suspension with 70μM biphenyl.

Viability of the suspension of the isolated hepatocytes was 75–80%. The cells were characterized by the maintenance of the structure of the plasma membrane, cell organelles (Fig. 1). After freezing of the suspension of hepatocytes both according to modes 1 and 2 the impairments in the structural organization were incompatible with the processes of their vital activity. Most cells are destroyed, debris with hyperchromic nuclei is often found. Single survived cells are deformed, their shapes are unusual, and the cytoplasm is dense. Mitochondria of these hepatocytes are increased in size, deformed, their outer membranes and crysts are virtually missing. Extensive ruptures of the inner membranes hydration-induced lightening of the matrix are also observed.

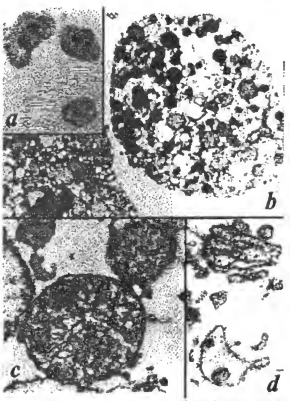

Fig. 1. Morphological survival of the hepatocytes, isolated from the cadaver liver of adult human donors (hematoxylin, eosin): *a* – X400, *b* – X9500, *c* – X36200, *d* – X64500, reduction during reproduction was 3/5

A comparative study of the functional activity of the native hepatocytes of cadavers and human fetuses showed some age-dependent peculiarities. A prolongation of the terms of development of the fetuses from 16 to 26 weeks brought about a tendency towards rising the level of endogenous respiration and reducing stimulation of the respiratory activity by exogenous succinate in the isolated thawed cells.

Table 1

Effect of the mode of freezing, type and concentration of cryoprotectant on the morphofunctional properties of human fetal hepatocytes (26 weeks of development), n=5–7

Experimental conditions	Parameter under study		
	Rate of endogenous respiration, nM O_2/min/10^6 cells	Factor of stimulation of endogenous respiration by succinate	Viability, trypan blue staining, % from control
Control	10,3±1,2	1,24±0,1	75
In the medium without cryoprotectant			
Mode 1	4.3±0.18	2.4±0.18	35
Mode 2	5.0±0.1	2.6±0.2	35
In the medium with cryoprotectants			
Mode 1+10%-cryoprotectant			
Glycerol	6.47±0.2	2.20±0.15	65
DMSO	7.04±0.3	2.14±0.14	68
Mode 2+3%-cryoprotectant			
Glycerol	6.1±0.2	2.20±0.15	70
DMSO	6.5±0.1	2.03±0.10	65
Mode 2+10%-cryoprotectant			
Glycerol	6.2±0.2	2.20±0.10	65
DMSO	6.4±0.4	2.45±0.20	68

Note: $p < 0.05$ as compared with control.

Investigation of the influence of the rates of freezing demonstrated an inhibition of the hepatocyte ability of every studied group to eliminate a vital stain, in-

corporate labeled amino acids into summary proteins, as well as acceleration of the oxidation rate of the exogenous succinate, which testifies to the impairment in the integrity of the plasma membrane and alteration of its barrier properties (Table 1). Incorporation of the labeled precursors into summary proteins of the thawed hepatocytes of human fetuses, aged 24–26 weeks of development, and cadavers was inhibited considerably and showed poor dependence on the rate of freezing, comprising on average 30% of the control values (Table 2).

Table 2

Effect of the mode of freezing and cryoprotectants on morphofunctional properties of cadaveric hepatocytes, n=7–10

Mode of freezing		Concentration of cryoprotectant, %	Viability of hepatocytes, %	Incorporation of amino acids into summary proteins of hepatocytes, pulses/min GS cells
Mode 1	DMSO	10	75	36230±96
	Glycerol	10	72	28253±100
Mode 2	DMSO	3	68	39925±120
	Glycerol	3	60	27421±91
	DMSO	10	72	39000±112
	Glycerol	10	60	28006±120

The ability to capture xenobiotic biphenyl by cadaveric hepatocytes after freezing them according to mode 1 in the medium without cryoprotectant was reduced on average by 50%. When accelerating the rate of freezing (mode 2) this index increased by 10% and did not differ from the values, obtained with this rate of freezing for human fetal hepatocytes aged 24–26 weeks of development (Fig. 2).

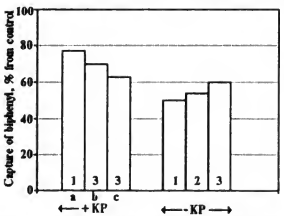

Fig. 2. Effect of the rate of freezing, type and concentration of the cryoprotectant on capture of biphenyl by allogenic hepatocytes: 1 – two-stage, 2 – 200 C/min, 3 – 16000 C/min; *a* – 10%-DMSO, *b* – 3%-DMSO, *c* – 3%-glycerol; KP – cryoprotectant

With the aim of protecting hepatocytes during low temperature preservation the authors used two approaches: acceleration of the rate of freezing in the one-stage modes and application of combined rates at the stages of freezing with involvement of a temperature stop. Acceleration of the rate of freezing results in the formation of the finely granular ice structure, weakening of action of such damaging factors as dehydration, dehydration of macromolecules, a rise in the concentration of electrolytes and other solutes, slow injury of cells by the intracellular ice, etc.

Freezing with the rate of 16000 °C/min makes it possible to reduce the effective concentration of the cryoprotectant down to 3%. If 3%-DMSO was present in the medium for freezing of hepatocytes, then the indices of incorporation of amino acids into summary proteins and capture of biphenyl were maintained at a higher level (Table 2, Fig. 2) as compared with 3%-glycerol. A rise in the DMSO concentration up to 10% in the medium and a change in the mode of freezing did not produce additional protective effect: viability of the thawed hepatocytes was practically similar after freezing according to both modes.

The analysis of changes in the functional activity of hepatocytes from human fetuses of 24–26 weeks of development after freezing according to the modes 1 and 2 showed that application of a 3%-glycerol or 3%-DMSO during one-stage suprarapid freezing provides for the same effect as regards to the studied parameters, as that of a two-stage freezing in the medium with 10%-DMSO. In fetal hepatocytes, frozen according to mode 2, viability was maintained at a level

similar to that after cooling according to mode 1, however, incorporation of the labeled amino acids and the rate of endogenous respiration were significantly higher, as compared with the hepatocytes, frozen according to mode 1 (Table 1). A stronger manifested cryosusceptibility of hepatocytes, isolated from human fetuses, should be mentioned as compared with that of cadaveric liver hepatocytes.

Thus, the results obtained testify to the fact that in the isolated hepatocytes, frozen in the medium with cryoprotectants according to the described modes, the ability to of the subcellular structures (e.g. mitochondria and endoplasmic reticulum) to interact is maintained, which is very important for clinical application of hepatocytes, since it is impossible in the injured cells. In particular, a directed regulation of the processes of biotransformation of xenobiotics is maintained, which provides for enhancement or weakening of the activity of microsomal oxidation. It is of special importance since the first stages of metabolism of some xenobiotics [1] are known to occur in the cytosol, while intermediate products are metabolized in mitochondria. It follows from the above that one of the criteria of applicability of hepatocyte suspension to cryopreservation and subsequent clinical usage should be a high level of viability of hepatocytes, which may be achieved only when all the membrane structures of the cells, which are involved into such important processes as bioenergetics, protein biosynthesis, xenobiotic detoxication, remain intact.

Reliability of the developed methods of harvesting and cryopreservation of human fetal and cadaveric hepatocytes with regards to the maintenance of their structure-functional properties was supported not only by experimental investigations, but by the efficiency of their practical application for extracorporal treatment of hepatic insufficiency in 69 patients (poisoning by mushrooms and hepatotropic poisons – CCl_4, dichlorethanol, etc.) with fixation of hepatocytes on the semipermeable membrane (175 cell dialyses) [13]. At the same time the observed [8] higher efficiency of conducting "cell" therapy with hepatocytes as compared with other detoxication methods requires accurate and detailed knowledge on the mechanism of their action, since as reported elsewhere [3], it is provided for not only by a high sorption ability of the cells, but also by some other mechanisms which haven't been "deciphered" yet.

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Н.П.СУББОТА

ФУНКЦІОНАЛЬНИЙ СТАН НАТИВНИХ І КРІОКОНСЕРВОВАНИХ АЛЛОГЕПАТОЦИТІВ, ЯКІ ПРИЗНАЧЕНІ ДО КЛІНІЧНОГО ВИКОРИСТАННЯ

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Вивчені структурний стан, дихальна та білоксинтезуюча активності, захват ксенобіотика біфенила нативними і кріоконсервованими гепатоцитами печінки плодів людини і кадаверів, які призначені до клінічного використання. Розроблені методи кріоконсервування гепатоцитів у середовищі з низькими концентраціями протекторів без зменшення їх життєздатності.

Н.П.СУББОТА

ФУНКЦИОНАЛЬНОЕ СОСТОЯНИЕ НАТИВНЫХ И КРИОКОНСЕРВИРОВАННЫХ АЛЛОГЕПАТОЦИТОВ, ПРЕДНАЗНАЧЕННЫХ ДЛЯ КЛИНИЧЕСКОГО ИСПОЛЬЗОВАНИЯ

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Изучены структурное состояние, дыхательная и белоксинтезирующая активность, захват ксенобиотика бифенила нативными и криоконсервированными гепатоцитами печени плодов человека и кадаверов, предназначенными для клинического использования. Разработаны методы криоконсервирования гепатоцитов в среде с низкими концентрациями протекторов без уменьшения их жизнеспособности.

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CRYOPROTECTIVE PROPERTIES OF POLYOLS AND THEIR DERIVATIVES DURING FREEZING OF PLATELETS

Cryoprotective properties of 1.2-propanediol and alkyl-, etoxy-derivatives of a classical cryoprotectant glycerol were studied during freezing of platelets. It was found that together with glycerol the highest cryoprotective ability was characteristic of 1.2-propanediol, 1-monomethyl ether of glycerol and oxyethylated glycerol (OEG, n=5) among the studied substances.

There are two approaches to solving the problem of platelet cryopreservation: on the one hand, it is a search for the conditions, providing for a reduction in cytotoxicity and enhancement of cryoprotective action of traditional cryoprotectants, on the other – a search for new cryoprotectants, possessing low toxicity and sufficient cryoprotective efficiency towards platelets. The authors applied a complex approach to the search for new cryoprotectants, which involves a directed synthesis of new substances using chemical modifications of the known cryoprotectants, study of their physical-chemical and cryoprotective properties, as well as of the nature and peculiarities of their influence on a cryobiological system [2, 3]. The aim of the present study was to investigate cryoprotective properties of 1.2-propanediol and derivatives of a classical cryoprotectant glycerol during freezing of platelets.

Human platelets, harvested by differential centrifugation into plastic bags of a 'Hemakon' type with a glugicir preservative, were used in experiments. The following substances were studied out of the polyol series: 1.2-propanediol (1.2-PD), glycerol, i.e. the substances which are commercially produced, 1-monomethyl (1-MMEG), 1.3-dimethyl (1.3-DMEG), 1.2-dimethyl (1.2-DMEG), 1.2-diethyl (1.2-DEEG), 1.3-diethyl (1.3-DEEG) ethers of glycerol, oxyethylated glycerol with the degree of polymerization of n=5 (OEG, n=5) – the substances, produced at the Department of Cryoprotectants of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of the Ukraine, Kharkov, under the supervision of L.A.Khanina, Senior Research Fellow. The substances were prepared on the plasma *ex tempore* and were mixed with a platelet concentrate in a 1:1 ratio and finite concentrations of 2.5, 5 and 7.5%. The time of exposure was 15–60 min. The samples of the platelet concentrate were frozen in polyethylene ampoules with the volumes of 1.5 ml at the rates of 1, 10, 20 and 30 °C/min down to −70 °C with subsequent immersion into liquid nitrogen. The frozen samples were thawed on water bath at 37 °C. Functional integrity of platelets was studied after preliminary elimination of the substances according to the following features: cell count in a Goryayev's chamber, morphological control with a phase-contrast microscope [6], determination of a retractile activity [2], ADP-induced aggregation (photometric method), responses to hypothermic stress (RHS) [4]. A test, based on the measurement of the optical density of the platelet suspension with protective solutions prior to freezing and after thawing, was used in the study of platelet cryopreservation [5].

Table 1

The effect of substances of a polyol class on the indices of the morphofunctional activity of platelets (30-min exposure, 22 °C; M±m, n=11)

Substances	Concentration of the substance, %	The indices under study, %				
		Amount	Retraction	Aggregation, ADP-inductor (20 mM)	RHS	Content of discoid shapes,
Control	0	100	83±3	71±4	81±3	79±7
1.2-PD	2.5	98±2	80±2	65±3	75±3	73±3
	5.0	97±3	78±2	62±3	72±4	69±5
	7.5	95±3	75±3	59±4*	69±4	67±4
Glycerol	2.5	94±2	78±2	61±5	73±2	70±4
	5.0	92±2	75±4	65±4	61±4*	55±7*
	7.5	86±3*	68±3*	57±4*	47±6*	49±8*
1-MMEG	2.5	96±2	76±3	60±5	75±3	72±4
	5.0	95±2	74±4	58±4*	63±2*	64±5
	7.5	83±3*	69±4*	44±5*	57±4*	55±6*
OEG (n=5)	2.5	95±2	78±4	59±5	62±4*	68±5
	5.0	90±4	69±4*	50±4*	50±3*	58±5*
	7.5	87±3*	66±3*	32±4*	33±3*	50±7*
1.3-DMEG	2.5	96±2	67±2*	49±5*	36±3*	70±5
	5.0	93±3	58±3*	36±5*	24±4*	52±6*
	7.5	80±3*	52±3*	22±4*	15±3*	39±8*
1.2-DMEG	2.5	89±2*	42±2*	32±4*	20±3*	32±8*
	5.0	93±3*	0	0	0	0
	7.5	68±3*	0	0	0	0
1-MMEG	2.5	87±2*	45±2*	29±4*	15±3*	30±5*
	5.0	80±3*	25±3*	14±3*	12±3*	0
	7.5	75±3*	0	0	0	0
1.3-DEEG	2.5	90±3*	42±3*	25±3*	20±3*	27±6*
	5.0	86±3*	20±3*	22±5*	10±3*	0
	7.5	80±3*	0	0	0	0
1.2-DEEG	2.5	83±4*	25±4*	14±5*	10±5*	18±5*
	5.0	75±3*	0	0	0	0
	7.5	63±3*	0	0	0	0

Note: * $p < 0.05$ as compared with control.

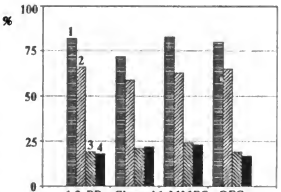
When studying cryoprotective properties of new substances it is important to investigate their cytotoxicity at the stage of exposure, which allows to select the substances with potential cryoprotective properties. The results of these studies are submitted in Table 1. It was found that out of 9 studied compounds the least manifested effect on the specific functions of platelets in the range of concentrations of 2.5–7.5% was exercised by 1.2-PD, glycerol, 1-MMEG, and OEG (n=5). Such features as the total amount of cells, retractile activity, were at sufficiently high level (80–98%) as compared with the initial value, independently of the concentration of these substances and time of exposure (15–60 min). At the same time maintenance of a discoid shape, characteristic of the intact platelets, changes in the response to hypotonic stress and aggregation properties of platelets were of a concentration-dependent nature. It should be noted that it was the maintenance of this discoid shape and the extent of the response to hypotonic stress which are the most information-producing indices *in vitro*, describing morphofunctional integrity of platelets. Following exposure with 2.5%-solutions of glycerol, 1.2-PD, 1-MMEG, OEG (n =5) the content of the discoid shapes in the samples was not changed as compared with control. A rise in the concentration of substances in the cell suspension reduced the amount of discoid shapes due to the appearance of the branch-like and spheric platelets. A significant lowering of the extent of the response to hypotonic stress, and aggregation were observed after increasing the concentrations up to 5 and 7.5%. It was found that the solutions, which contain 1.3-DMEG, strongly inhibited functional activity of platelets, especially the ability to respond to hypotonic stress. At the same time the amount of discoid plates after a contact with a 2.5%-solution of 1.3-DMEG did not differ from control. The most manifested inhibiting action of the specific functions was exercised by the solutions: 1.2-DMEG, 1-MMEG, 1.3-DEEG and 1.2-DEEG. Already after a 15-min exposure with these substances the total amount of platelets was reduced. the retractile activity was decreased by 30–50% on average after exposure with 2.5%-concentration of the solutions, while 5%- and 7.5%-concentrations of 1.2-DMEG, 1.2-DEEG and 7.5%-concentrations of 1-MEEG, 1.3-DEEG brought about the irreversible inhibition of the given function. Simultaneously the platelets lost the ability for an ADP-induced aggregation and response to hypotonic stress. After a 60-min exposure the action of these substances was enhanced, which was manifested as the reduction of the total amount of cells and retractile activity, as well as the irreversible inhibition of aggregation and ability to respond to hypotonic stress when using 2.5%-concentrations of the solutions.

The data, obtained when studying cytotoxicity of the substances of the same series with the structures, gradually getting more and more complicated, testify to the fact that prolongation of the length and a rise in the amount of alkyl radicals increase the cytotoxicity of the substances. The authors established a direct dependence between the cytotoxicity and hydrophobic nature of these compounds, which was determined by the factor of ditribution of the substances in an octanol-water system.

A primary screening of the cryoprotective activity of the studied compounds (determination of the total amount of cells, retractile activity, as well as the measurement of the optical density of the syspensions with protective solutions prior to and after freezing) showed that, beside glycerol, 1.2-PD and 1-MMEG in a 2.5%-finite concentration at the rate of cooling of 1–10 °C/min exercised a highest cryoprotective action (Table 2).

When using 5%-concentrations of 1.2-PD, 1-MMEG, and 2.5%-concentration of glycerol, the indices of the platelet survival are reduced. After freezing the platelet concentrates under protection of 1.3-DMEG, 1.2-DMEG, 1-MMEG, 1.3-DEEG, 1.2-DEEG with the rates of 1 and 10 °C/min the total amount of cells was

within the range of 65–80%. However, low indices of the optical density, retraction of the plasma clot, and a complete loss of the retractile activity when using 1.3-DEEG and 1.2-DEEG, do not allow to assume that the given compounds demonstrate cryoprotective properties already at the stage of screening. With the aim of determining the efficiency of cryopreservation of platelets with the substances under study the authors investigated survival of the basic specific functions of blood plates. When freezing platelets with the rates of 1 and 10 °C/min under protection of 5%-concentration of glycerol, on the average 70% of platelets were identified, while under protection of a 2.5-% concentration of 1.2-PD and 1-MMEG this figure equaled 80% on the average, and an extremely labile, highly specific function of the platelets was maintained, namely, that of the aggregation activity. There were no significant differences in the level of the ADP-induced aggregation when using glycerol, 1.2-PD and 1-MMEG, and its values were as follows: 21±4 for glycerol, 18±3 for 1.2-PD, and 24±3 for 1-MMEG as compared with the original level. When freezing platelets with glycerol, 1/2-PD and 1-MMEG with the rates of 1 and 10 °C/min the response to hypotonic stress was maintained; the best results were obtained after cryopreservation with a 5%-glycerol solution and 2.5%-solutions of 1.2-PD and 1-MMEG, and comprised 22±3, 19±4 and 23±3%, respectively (Fig.). Acceleration of the rate of cooling reduced the indices of the response to hypotonic stress, while cooling at a rate of 30 °C/min under protection of 1.2-PD and 1-MMEG completely eliminated the ability of platelets to respond to hypotonic stress.



The indices of the functional integrity of platelets after freezing under protection of polyols: *1* – amount of cells, *2* – retraction, *3* – aggregation, *4* – response to hypotonic stress

Table 2

Survival of platelets, cryopreserved under protection of the substances of the polyol series under various modes of cooling (M±m; n=9)

Substances	Concentration of the substance, %	The indices under study, %								
		Amount of cells			Retraction			Optical density		
		Rate of cooling, °C/мин								
		1	10	20	1	10	20	1	10	20
1.2-PD	2.5	82±3	80±3	72±2*	66±4*	61±3*	53±4*	91±5	89±3	76±4*
	5.0	87±4	83±2	73±3	52±5	50±4	48±3	80±3	78±3	75±3
Glycerol	2.5	60±3	65±4	72±2*	39±6	41±3	30±4	78±3	76±4	70±3*
	5.0	72±4*	72±3*	76±3	59±3*	52±4*	48±5*	83±2	81±3	78±3
1-MMEG	2.5	83±2	81±2	71±4*	63±4	59±3	47±4*	85±3	80±3	71±2*
	5.0	85±4	80±3	68±3	51±5	49±4	41±5	79±4	76±5	70±3
1.3-DMEG	2.5	80±4*	76±2	65±4*	27±4*	25±3*	17±2*	76±3	73±4	61±2*
	5.0	69±3	73±4	80±3	15±3	10±2	0	73±2	70±3	66±4
1.2-DMEG	2.5	70±3	68±4	60±4*	20±4	24±3	30±5	72±3	70±5	58±5*
	5.0	79±2	75±3	67±3*	0	0	0	70±2	67±4	58±4*
1-MMEG	2.5	69±3	66±2	59±4*	20±3	23±5	0	73±3	71±4	63±5
	5.0	76±4	70±3	65±5	0	0	0	70±2	66±3	60±3*
1.3-DEEG	2.5	71±2	67±3	60±3*	0	0	0	64±4	60±6	55±4
	5.0	78±3	73±2	66±2*	0	0	0	61±5	59±6	53±3
1.2-DEEG	2.5	63±4	62±3	52±4*	0	0	0	62±4	60±4	52±6
	5.0	70±3	68±2	57±3*	0	0	0	58±5	57±6	50±5
OEG (n=5)	2.5	76±3	81±4	71±4	54±4	65±4*	50±3	81±3	78±4	70±3*
	5.0	89±4	80±3	69±3	51±5	49±4*	33±4	82±3	77±5	75±4

Note: * – $p < 0.05$, the differences are significant with the rate of freezing of 1 °C/min; * – $p < 0.05$, the differences are significant between the figures for 2.5 and 5%-concentrations of the substances.

Since all the experiments with platelets were conducted for the first time, the authors investigated functional integrity of platelets after freezing with solutions of 1.3-, 1.2-DMEG, 1-MMEG and 1.3-, 1.2-DEEG. It turned out that independently of the rate of cooling platelets irreversibly lost their specific functions: aggregation, response to hypotonic stress. Morphologically predominant were spheres with spine-like processes. The data obtained support the absence of cryoprotective properties towards platelets in the above substances, thus making it possible to recommend the usage of the primary screening only for simplifying and shortening investigations of the type. A few words are to be said about the oxyethylated glycerol – OEG (n=5), which is a cryoprotectant with the exocellular mechanism of action. It was found that cryopreservation of platelet concentrates with protective solutions, containing 5 and 10%-concentrations of OEG (n=5), permits to preserve 70–80% of platelets. The highest values of retraction, aggregation, response to hypotonic stress were obtained when cooling platelet concentrates under protection of a 5%-solution of OEG (n=5) with the rate of cooling of 10 °C/min, and were as follows respectively: 65±3, 19±4 and 17±4.

Thus, the investigations conducted made it possible to identify a series of new substances, which seem to be promising for application as cryoprotectants when freezing platelets: 1.2-propanediol, 1-monomethyl ether of glycerol, oxyethylated glycerol with the extent of polymerization of n=5.

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КРІОПРОТЕКТОРНІ ВЛАСТИВОСТІ ПОЛІОЛІВ ТА ЇХ ПОХІДНИХ ПРИ ЗАМОРОЖУВАННІ ТРОМБОЦИТІВ

Інститут проблем кріобіології і кріомедицини НАН України, м. Харків

Вивчались кріопротекторні властивості 1,2-пропандіолу і алкіл-, етокси-похідних гліцерину при заморожуванні тромбоцитів. Шз усіх досліджених речовин, окрім гліцерину, найбільш значними кріозахисними властивостями відзначались 1,2-пропардіол, 1-монометиловий ефір гліцерину та оксиетильований гліцерин (ОЕГ, n=5).

А.В.НИКОЛЕНКО, А.М.КОМПАНИЕЦ, В.И.ЛУГОВОЙ

КРИОПРОТЕКТОРНЫЕ СВОЙСТВА ПОЛИОЛОВ И ИХ ПРОИЗВОДНЫХ ПРИ ЗАМОРАЖИВАНИИ ТРОМБОЦИТОВ

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Исследовались криопротекторные свойства 1,2-пропандиола и алкил-, этокси-производных классического криопротектора глицерина при замораживании тромбоцитов. Из числа исследованных веществ наряду с глицерином выраженными криозащитными свойствами

обладают 1,2-пропандиол, 1-монометиловый эфир глицерина и оксиэтилированный глицерин (ОЭГ, n=5).

CRYOMEDICINE

UDC 47.043:615.832.9:591.112.2:57.084.1

S.M.Romanets

STUDY OF TEMPERATURE DEPENDENCE OF CHANGES IN ELECTROPHYSIOLOGICAL INDICES OF RAT HEART DURING CRANIOCEREBRAL HYPOTHERMIA

The effects of rat body temperature changing during craniocerebral hypothermia (CCH) and subsequent thawing on the duration of the processes of behavior and propagation of excitation in the myocardium, time of depolarization and repolarization of myocytes, and the duration of the electric systole and diastole of the myocardium were studied. The authors determined the rules of changes in the indices under study in the time course of cooling down to the rectal temperature of 28 °C and thawing. It was found that the duration of the electrophysiological indices of the rat heart during cooling increases differently, while it is reduced during thawing, though it is not completely restored even at 37 °C.

Currently CCH is widely used in clinical practice. This method was effective in treatment of the psychic neurotic disorders [5], chronic alcoholism [2], infertility [3], acute poisoning [1], etc. Up-to-now the process of regulation of the heart activity after such a cooling have been studied [4]. Earlier it was reported [6] that after cooling of the isolated heart of the dog from 35 to 25 °C the nervous, especially sympathetic regulation, is impaired, and a manifested inhibition of the chronotropic self-regulation of contractile activity of the myocardium becomes ebident. At the same time such a hypothermic treatment affected in the least the rate of conducting excitation through the auricles. However, the available reports on the detailed studies of the peculiarities of the electrophysiological features of the rat myocardium in the time course of CCH and warming are few. That is why the aim of the given study was to investigate a temperature dependence of the changes in the complex of the electrophysiological features of the rat heart during CCH with subsequent warming.

White outbred male and female rats with the body weights of 200–300 g were used in experiments. CCH was realized by cooling of the outer integuments of the head by a coolant (ice) with direct contact down to the rectal temperature of 28 °C. Upon achieving the required temperature the cold exposure was terminated, and the animals were thawed at the ambient temperature of 30–35 °C. In the course of experimentation the authors measured the temperature of the rat body in the rectum using a copper-constantan thermocouple. For blocking the reflectory thermoregulatory responses of the organism the animals were anaesthesized with a mixture of sodium thiopental (Medical Preparations Plant, Kiev, Ukraine) and sodium oxybutyrate (Chemical Pharmaceutical Plant №6, Riga, Latvia) at a dose of 30 and 100 mg/kg of body weight, respectively. The electrocardiogram (ECG) was registered with a EK1K-01 at a second standard lead. The ECG was taken during cooling and warming of the animal at every degree of the body temperature changing. When analyzing the ECG curves, the authors calculated the prolongation of the intervals: from the start of the wave P to the start of the wave Q (time of conducting excitation from the auricles to the ventricles), from the beginning of the segment P to its end (time of propagation of excitation through the auricle, and the time of depolarization of the auricle), from the start of the wave Q and to the end of the wave S (time of depolarization of the ventricles), from the start of the wave Q and to the end of the wave T (prolongation of the electrical systole of the

ventricles), from the start of the wave T to its end (time of repolarization of the myocytes of the ventricles) [7, 8], from the start of the wave (peak) Q to the start of the wave P of the next cycle (electrical diastole of the auricle), from the start of the wave T to the start of the wave (peak) Q of the next cycle (electrical diastole of the ventricles), from the end of the wave T to the start of the wave P of the next cycle (time of the total diastole of the myocardium) and interval RR (duration of the heart cycle) [7, 8].

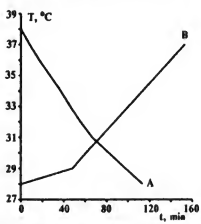

Fig. 1. Dynamics of temperature changes of the rat body during cooling (A) and warming (B)

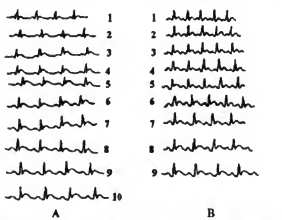

Fig. 2. Electrocardiograms of rat during cooling (A) and warming (B). Body temperature, °C: 1 – 37, 2 – 36, 3 – 35, 4 – 34, 5 – 33, 6 – 32, 7 – 31, 8 – 30, 9 – 29, 10 – 28

Fig.1 shows the dynamics of the animal temperature lowering during cooling and warming. It follows from the diagram that the process of cooling occurred regularly down to 31 °C with the rate of 0.09 °C/min and further with the rate of 0.06 °C/min down to 28 °C. Thawing of the animals was performed in two stages. From 28 C to 29 °C the animal was warmed slowly at a rate of 0.02 °C. After that thawing occurred regularly and its rate accelerated up to 0.08 °C/min.

Fig. 2 displays the ECG, taken from the animals prior to the start of the hypothermic exposure, as well as in the process of cooling and warming. Considerable changes in the electrophysiological pattern of the heart muscle of rats during CCH and warming are evident as compared with normothermia. The results of the ECG processing showed that the heart rate (HR) is reduced by 1.7-fold during cooling (Fig. 3). It is evident from Fig. 4 that such a slowing down of the rhythm relates, mainly, to the prolongation of the heart cycle, in the first place, of the myocardium diastole (TP). Here the duration of the total myocardium diastole (TP) of the rats remains practically unchanged until the rectal temperature of 31 °C is achieved, and then sharply increases by 2.1-fold. It should be noted that other electrophysiological indices of the rat heart (time of propagation of the excitation, duration of the processes of depolarization and repolarization of the auricles and ventricles) increase their values in the temperature range of 37 to 28 °C (Fig. 4, *5*). In particular, the duration of the electric diastole of the auricles (QP) is prolonged by 1.7-fold, while the dynamics of its changes is similar to the dynamics of changes in the duration of the total myocardium diastole (TP) (Fig. 4). It was shown that the time of conducting excitation from the auricles to the ventricles (PQ), duration of the electrical systole (QT) and diastole (TQ) of the ventricles and the time of repolarization of the ventricles (T) increase by 1.5-fold (Fig. 4, *5*). At the same time the duration of the ventricle depolarization (QS) increases insignificantly (Fig. *5*), while the time of propagation of excitation along the auricles is not altered throughout the whole process of cooling.

As the thawing of the animals went on, the HR gradually accelerated, overtaking the normal rate. Upon complete thawing this value exceeds the HR of the uncooled rats by 1.4-fold (Fig. 3). Such a change in the heart rhythm relates to a

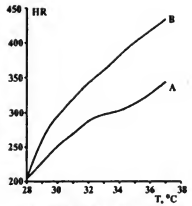

Fig. 3. Dynamics of changes in the HR of rats during cooling (A) and warming (B)

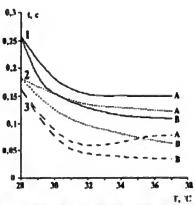

Fig. 4. Dynamics of diastolic characteristics of the rat heart cycle during cooling (A) and warming (B). Duration of: 1 – electric diastole of the auricles; 2 – electric diastole of the ventricles; 3 – total diastole of the myocardium

shortening of the heart cycle by 1.4-fold. When analyzing the ECG, some peculiarities are to be reported. All the time intervals of the heart cycle during thawing are reduced regularly, with the exception of the time of conducting excitation along the auricle, which remains the same. By the end of thawing the values of these parameters do not return to the normal level. The exception is the time (duration) of depolarization of the ventricles, the magnitude of which towards the end of thawing is restored. A degree of reduction of the heart cycle parameters during warming is not similar for different intervals, and it is most pronounced for the time (duration) of the total myocardium diastole (TP) (Fig. 4, 5).

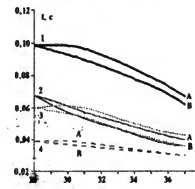

Fig. 5. Dynamics of the systolic characteristics of the rat heart during cooling (A) and warming (B). Duration of: 1 – electrical systole of the ventricles, 2 – conduction of excitation from the auricle to the ventricles; 3 – repolarization of the ventricle myocyte; 4 – depolarization of ventricles

Judging from the results obtained one can assume that the nature of changes in the membrane processes in the heart cells is different. Fig.5 testifies to a gradual prolongation of the action potential during cooling, and Fig. 4 supports relative stability of the rest potential until 30–32 °C, after which it is rapidly disturbed. It can be also assumed that certain physiological mechanisms of regulation of the heart rate also undergo changes. The results obtained permit to conclude on the peculiarities of changes in the systolic and diastolic parameters of the rat myocardium during CCH and warming. It was found that the duration of the electrophysiological parameters of the rat heart during cooling is prolonged to a different degree, and it is shortened during warming, and is not restored completely even at 37 °C. The systolic characteristics change in a monotonous way, while the diastolic ones are altered insignificantly at 32–30 °C, and then an abrupt change occurs.

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С.М.РОМАНЕЦЬ

ВИВЧЕННЯ ТЕМПЕРАТУРНОЇ ЗАЛЕЖНОСТІ ЗМІН ЕЛЕКТРОФІЗІОЛОГІЧНИХ ПОКАЗНИКІВ СЕРЦЯ ЩУРІВ ПІД ЧАС КРАНІОЦЕРЕБРАЛЬНОЇ ГІПОТЕРМІЇ

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Вівчено вплив зміни температури тіла щурів під час краніоцеребральної гіпотермії та подальшого відігрівання на тривалість процесів проведення та росповсюдження збудження у міокарді, час деполяризації та реполяризації міоцитів, а також тривалість електричної систоли і діастоли міокарду щурів. Вивчені закономірності зміни показників в динаміці охолодження до ректальної температури 28 °С та відігрівання. Встановлено, що тривалість електрофізіологічних показників серця щурів під час охолодження збільшується неоднаково, а під час відігрівання зменшується, не відновлюючись повністю навіть при 37 °С.

С.М.РОМАНЕЦ

ИЗУЧЕНИЕ ТЕМПЕРАТУРНОЙ ЗАВИСИМОСТИ ИЗМЕНЕНИЙ ЭЛЕКТРОФИЗИОЛОГИЧЕСКИХ ПОКАЗАТЕЛЕЙ СЕРДЦА КРЫС ПРИ КРАНИОЦЕРЕБРАЛЬНОЙ ГИПОТЕРМИИ

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Изучено влияние изменения температуры тела крыс при краниоцеребральной гипотермии (КЦГ) и последующем отогреве на длительность процессов проведения и распространения возбуждения в миокарде, время деполяризации и реполяризации миоцитов, а также длительность электрической систолы и диастолы миокарда. Определены закономерности изменения исследуемых показателей в динамике охлаждения до ректальной температуры 28 °С и отогрева. Установлено, что длительность электрофизиологических показателей сердца крыс при охлаждении увеличивается в неодинаковой степени, а при отогреве уменьшается, не восстанавливаясь полностью даже при 37 °С.

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V.A.Chlpizhenko

THE EFFECT OF LOCAL HYPOTHERMIA ON THE KINETIC PARAMETERS OF BINDING AND DENSITY OF β-ADRENORECEPTORS, AND THE CONTENT OF CYCLIC NUCLEOTIDES IN THE SKIN WITH ALLERGIC CONTACT DERMATITIS IN THE EXPERIMENT

The authors studied the action of local hypothermia (cooling of the injured limb in the air chamber of the hypotherm down to the temperature of 22–24 °C of the skin for 1 hour) on the kinetic parameters of binding and density of β-adrenoreceptors, and the level of the skin cyclic nucleotides with allergic contact dermatitis on 2.4-dinitrochlorbenzene. They speculate on the possible application of local hypothermia for external application for treating allergic dermatitis.

In the mechanism of allergic dermatoses a major part is played by the impairments in the vegetative nervous system, which actively participates in the stabilization of the structure of cells and tissues, in the regulation of their functional activity via intracellular regulatory mechanisms. For example, the activation of the regulatory chain: sympatic nervous system – local release of catecholamines – stimulation of β-adrenoreceptors (AR) and conjugated adenylate cyclase – accumulation of the intracellular cyclic adenosine-3',5'-monophosphate – results in the reduction of excretion of histamine, serotonin, kinins, lysosomal enzymes by the cells of blood and skin, which take part in the development of allergic inflammation [2, 4, 7]. Apparently, when affecting separate links of the described regulatory chain, e.g. AR, it is possible to influence the occurrence of the skin allergic responses. Still, there are some reports on the dependence of the functional state of AR on temperature [1, 9, 11, 13]. Considering the above, the authors aimed to investigate the action of local hypothermia on the kinetic parameters of binding and density of β-AR, as well as on the content of the cyclic adenosine-3',5'-monophosphate (cAMP) and cyclic guanosine-3',5'-monophosphate (cAMP) in the skin of guinea pigs with the allergic contact dermatitis (ACD).

In 24 male white guinea pigs with the body weights of 350–400 g the ACD was reproduced on 2.4-dinitrochlorbenzen (DNCB). Sensibilization was carried out by a single application of 0.1 ml of 5%-solution of DNCB on the shaven portion of the femur skin, simultaneously applying 0.02 ml of 1%-aqueous solution of DNCB on seven other shaven portions of the *dorsum* skin. On the 14th day from the start of the experiment the femur skin was plastered with a permissible triggering dose of DNCB – 0.02 ml of 1%-solution. The next day on the place of application the ACD was developed [5]. In 12 animals the bioptates of the skin for investigation were collected from the foci immediately, while 12 other animals tolerated local cooling of posterior limbs (place of localization of the ACD) in the air chamber of a hypotherm down to the skin temperature of 22–24 °C (controlled by electrothermometer) for 1 hour, after which the injured skin was harvested for investigation. The skin of 10 intact guinea pigs served as control. With the aim of studying the binding of AR with ligands the preparations of the plasma membranes of the skin cells were used, obtained and purified as described elsewhere [6].

Binding of β-AR was performed using ^{3}H-dehydroalprenolol 06.-6 nM in the presence of 10 mM $MgCl_2$. A condition of the kinetic equilibrium was achieved in 20 min at the temperature of 25 °C. Separation of the bound and free ligand was conducted by a method of vacuum filtering through the fiberglass YF/c filters "Whatman". The value of the specific binding was determined by the difference between the level of binding in the absence and presence of the nonlabeled ligands. All the conditions of binding of β-AR were as described elsewhere [12]. The calculation of the kinetic parameters of the binding and content of AR was done with a PC on the basis of the analysis of the curves of binding in the Scatchard's coordinates. The kinetic parameters of binding – Kd (constant of dissociation of the AR-ligand complex) was expressed in nmol, while B_{max} (AR density) – in pmol/mg protein. At the same time the intracellular content of cAMP and cGMP was studied in the same skin samples. The latter was determined by a radioimmunologic method using standard kits TRK-432 and TRK-500, respectively (Amersham, England), and was expressed in pmol/g raw tissue. The results of investigations of the influence of the local hypothermia on Kd and B_{max} of β-adrenoreceptors of the guinea pig skin are submitted in Table 1.

It follows from Table 1 that the development of the ACD was followed by a rapid increase in Kd ($P<0.001$), while the growth of B_{max} was statistically insignificant ($P>0.1$). A single local hypothermic treatment resulted in a rapid reduction in Kd below the control level ($P<0.001$) and an insignificant decrease in B_{max},

Table 1

Kd of the complex β-AR-ligand and B_{max}-AR of the guinea pig skin

Statistical indices	Intact skin (control)	Skin with ACD prior to hypothermia	Skin with ACD after hypothermia
Kd, nmol			
n	10	12	12
M±m	2.21±0.2	6.17±0.64	1.22±0.14
P	–	< 0.001	< 0.001
B_{max}, nmol/mg			
n	10	12	12
M±m	0.094±0.015	0.127±0.013	0.075±0.017
P	–	> 0.1	> 0.5

which was statistically insignificant as compared with control (P>0.5) and significant as compared with a corresponding index prior to hypothermia (P<0.05). However, when studying any receptor with a method of radioligand binding it is expedient to reveal the correlation between the ligand binding and the extent of manifestation of its physiological activity, which is done to exclude the so-called "silent receptors". The latter are capable of both non-specific and specific binding of the ligand, though they do not mediate cell physiological response [3]. Physiological activity of the β-AR agonists may be judged upon by the level of the intracellular cAMP, since it is currently believed that there exists a close conjugation of the β_1- and β_2-AR and adenylate cyclase – the enzyme, which is responsible for the synthesis of cAMP [3, 8, 10]. Considering the above, the authors thought it expedient to determine the intracellular content of cAMP and cGMP in the same samples of the guinea pig skin (Table 2).

Table 2

Content of cAMP and cGMP in the skin of guinea pigs in pmol/g raw tissue

Statistical indices	Intact skin (control)	Skin with ACD prior to hypothermia	Skin with ACD after hypothermia
cAMP			
n	10	12	12
M±m	229.0±29.2	54.0±9.0	166.0±22.6
P	–	< 0.001	< 0.01
cGMP			
n	10	12	12
M±m	35.0±4.73	41.0±7.23	13.9±3.54
P	–	> 0.2	< 0.001

It follows from Table 2, with the development of the ACD the content of cAMP in the injured skin was rapidly reduced (P<0.001), while an increase in cGMP was statistically insignificant (P>0.2). Following a hypothermic treatment the level of cAMP was increased considerably as compared with the original level (P<0.001), though it did not achieve the normal limits (P<0.01). At the same time the level of cGMP was rapidly reduced below the normal level (P). The dynamics of the ratio cAMP/cGMP was even more informative. In the intact skin it comprised 6.5 on the average; during ACD it was considerably reduced down to 1.3 and rapidly increased above the normal level up to 11.9 after a conducted hypothermic treatment.

When comparing the Kd dynamics of the complex β-AR-ligand with the content of cAMP in the skin, a distinct inverse dependence was observed between the first and the subsequent two indices. For example, during the ACD development a rise in Kd by 2.8-fold on the average was accompanied by a simultaneous rise in the level of cAMP by 4.2-fold and a reduction of the ratio cAMP/cGMP by 5-fold. Following hypothermic treatment a reduction in Kd by 5.1-fold as compared with the original value was accompanied by a simultaneous rise in the cAMP content by 3.1-fold, and that of the ratio cAMP/cGMP – by 9.1-fold as compared with the original level.

Thus, a revealed considerable impairment in the functional activity of β-AR and intracellular content of the cyclic nucleotides in the injured skin make it possible to assume that the said impairments play a pathogenic role during ACD. Local skin hypothermia with ACD, on the one hand, rapidly increases a reduced affinity of GB-AR to a respective ligand, on the other hand, it results in the insignificant

reduction of the density of the receptors of this type. One may expect a rise in the event of a β-adrenergic stimulation, which is supported by a growth of the intracellular content of cAMP, and inhibition of liberation of some mediators of the allergic inflammation and lysosomal enzymes.

The results obtained permit to assume that local hypothermic treatment of the skin, while acting via β-AR and a system of cyclic nucleotides, may modify the occurrence of skin allergic responses. A similar assumption may serve as a theoretical substantiation for application of local hypothermia in the external therapy of the patients with allergodermatoses.

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ВПЛИВ МІСЦЕВОЇ ГІПОТЕРМІЇ НА КІНЕТИЧНІ ПАРАМЕТРИ ЗВ'ЯЗУВАННЯ І ЩІЛЬНІСТЬ β-АДРЕНОРЕЦЕПТОРІВ, А ТАКОЖ ВМІСТ ЦИКЛІЧНИХ НУКЛЕОТИДІВ ШКІРИ З АЛЕРГІЧНИМ КОНТАКТНИМ ДЕРМАТИТОМ У ЕКСПЕРИМЕНТІ

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Вивчався вплив місцевої гіпотермії (охолодження ураженої кінцівки у повітряній камері гіпотерма до температури поверхні шкіри 22–24 °С на протязі 1 г) на кінетичні параметри зв'язування і щільність β-адренорецепторів, а також рівень циклічних нуклеотидів шкіри з алергічним контактним дерматитом на 2,4-динітрохлорбензол (ДНХБ). Розглядається можливість застосування місцево гіпотермії для зовнішнього лікування алергічних дерматозів.

В.А.ЧИПИЖЕНКО

ВЛИЯНИЕ МЕСТНОЙ ГИПОТЕРМИИ НА КИНЕТИЧЕСКИЕ ПАРАМЕТРЫ СВЯЗЫВАНИЯ И ПЛОТНОСТЬ β-АДРЕНОРЕЦЕПТОРОВ, А ТАКЖЕ СОДЕРЖАНИЕ ЦИКЛИЧЕСКИХ НУКЛЕОТИДОВ КОЖИ С АЛЛЕРГИЧЕСКИМ КОНТАКТНЫМ ДЕРМАТИТОМ В ЭКСПЕРИМЕНТЕ

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Изучалось влияние местной гипотермии (охлаждение пораженной конечности в воздушной камере гипотерма до температуры поверхности кожи 22–24 °С в течение 1 ч) на кинетические параметры связывания и плотность β-адренорецепторов, а также уровень циклических нуклеотидов кожи с аллергическим контактным дерматитом на 2,4-динитрохлорбензол. Рассматривается возможность применения местной гипотермии для наружного лечения аллергических дерматозов.

BRIEF COMMUNICATIONS

UDC 57.043:611.36

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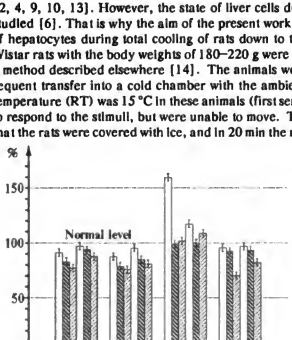
FINE STRUCTURE OF THE RAT LIVER CELLS DURING TOTAL COOLING

The responses of the organism, tissues and organs of warm-blooded animals and man to total cooling have been studied for many decades [1, 2, 7, 14]. A possibility of principle of the reversible cooling of homolothermic organisms was confirmed as long ago as in the past century, but until now a physiologically adequate method of a long-term maintenance of these states is still missing [3, 7, 8]. The interest to the idea of a reversible slowing down of the vital processes persists until nowadays. A direct action of cooling on the tissues of the organism may result in the development of stress reactions, which involve neurovegetative and hormonal systems. This, in turn, may bring about enhancement of heat production and metabolism [2, 4, 9, 10, 13]. However, the state of liver cells during profound total cooling has been insufficiently studied [6]. That is why the aim of the present work was to investigate the ultrastructural characteristics of hepatocytes during total cooling of rats down to the temperatures of 15, 9, 6 °C. Mature white male Wistar rats with the body weights of 180–220 g were used in experiments. The animals were cooled using a method described elsewhere [14]. The animals were placed into a case with a volume of 2 l with subsequent transfer into a cold chamber with the ambient temperature of 0 °C. Two hours later the rectal temperature (RT) was 15 °C in these animals (first series of experiments). Here the animals were still able to respond to the stimuli, but were unable to move. The heart rate (HR) was 60 beats per minute. After that the rats were covered with ice, and in 20 min the rectal temperature was 9 °C (second series of experiments), the respiration of the rats stopped, and HR did nor change significantly. Subsequent cooling of the rats for another 20 min lowered RT down to 6 °C, and the HR approached to zero (third series of experiments). The animals, kept at room temperature, served as control. After slaughtering the animals of the first, second and third groups, respectively, the livers of the cooled rats were rapidly extracted for subsequent studies. The authors conducted a histological control of the liver using a standard technique. For electron-microscopic investigations the liver fragments were fixed in a 2%-solution of glutaraldehyde and postfixed in a 1%-solution of OsO_4. Then the samples were dehydrated by acetone in an increasing concentration and embedded with epon-araldyte mixture according to a standard technique [12]. The ultrathin slices were prepared with a UPTM-6 ultramicrotom, contrasted with a saturated solution of uranyl acetate and plumbum citrate using a method described elsewhere [6]. The samples were visualized and their images were taken with a



Results of the PC-aided morphometric analysis of the fine structural parameters of hepatocytes during cooling down to 15 °C (abscissa–1, 4, 7, 10, 13, 16, 19, 22), to 9 °C (2, 5, 8, 11, 14, 17, 20, 23) and down to 6 °C (3, 6, 9, 12, 15, 18, 21, 24). The values of the area are of the following: nuclei 1–3; diffuse chromatin 7–9; compact chromatin 10–12; EPR 13–15; mitochondria 19–21. The values of the perimeter: nuclei 4–6; EPR 16–18; mitochondria 22–24. Along the ordinate axis are given the values of the perimeters, %

EMB-100 BR electron microscope with the accelerating voltage of 75 kV. The morphometric analysis of the cell organoids was performed with the help of a computerized system 'Morpho-Tools', designed and developed by Prof. A.S.Kaprelyants and A.N.Reylyan (Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of the Ukraine, Kharkov). For input of the images use was made of a K 6405 digitizer and PC CM1910 'Robotron'. A set of the applied programs of a 'Morpho-Tools' system enabled to calculate morphometric characteristics, their statistical processing and submission of the results in the digital and graphic forms. The results of the first series of experiments showed a routine architectonics of the liver cells at the microscopic level. The electron-microscopic analysis demonstrated that qualitatively the fine structure of hepatocytes is of a normal appearance and is characterized by the presence of the rounded mitochondria of various dimensions. Granular and smooth endoplasmic reticulum is presented in the form of cisterns with local expansions. The hepatocyte nuclei are of the rounded shape with regular contours. Finely dispersed chromatin is distributed along the whole nucleoplasm, and condensed chromatin is found in the form of small lumps along the nuclear membrane. Globular-fibrillar structure of the nuclei is not changed. The analysis of the morphometric indices of the intracellular structures of hepatocytes in the given series of experiments made it possible to observe a slight reduction in the area of the nuclei, as well as of diffuse and compact chromatin (Fig.).

There was found a tendency towards a decrease in the area of mitochondria and endoplasmic network. In the second series of experiments the electron-microscopic study revealed enlightening of the mitochondrial matrix, which has a finely granular structure. The nuclei of the rounded or ellipsoid shapes are reduced in size. The channels of the endoplasmic network are close to the normal shape, though sometimes the channels, placed in the vicinity of the mitochondria, are expanded. In some places the irregular pattern of the dislocation of ribosomes on the membranes of the endoplasmic reticulum is reported. The drops of fat are diffusively located throughout the whole cell. The morphometric indices during such type of treatment testify to a further reduction in the area and perimeter of the nuclei, area of the compact and dense chromatin, as well as of the decrease in the areas and parameters of the endoplasmic reticulums and lipids. In the third series of experiments the investigation of the fine structure indicates to the availability of the more manifested alterations in the organoids of hepatocytes of the rats. Compact chromatin was accumulated mainly on the inner surface of the nuclear membrane. The nuclei are visually reduced in size. The mitochondria were characterized by a manifested enlightening of the matrix and destruction of crysts. The lipid inclusions in the hepatocyte cytoplasm were found in small numbers. The morphometric data testify to a further reduction in the area and perimeter of the nuclei, endoplasmic network, compact chromatin, lipids. Thus, the investigations conducted allow to qualitatively and quantitatively assess structural rearrangements of the hepatocyte organoids during total cooling of various extent. The results of the visual analysis of transmission electron microscopy are supplemented and nicely correlate with the results of the morphometric studies. The hepatocyte organoids at various stages of profound cooling are characterized by a reduction in the area and perimeter of the nucleus, as well as by a decrease in the area of diffuse chromatin upon changing the RT from 15 to 9 °C. These values are slightly reduced when lowering the RT from 9 to 6 °C. At the same time the response of the compact chromatin is different. When the RT equals 15 °C or 9 °C, the differences are minimum, but further lowering down to 6 °C results in its rapid increase. The response of the mitochondria to profound cooling is manifested by the enlightening of the matrix of these organelles. And the most pronounced alterations are found during cooling down to 6 °C. When cooling the animals down to the rectal temperature of 15 and 9 °C the authors also observed changes in the fine structure of these organelles, which may be indicative of the initial stage of disintegration of the cell structures and functional disorder of the cell, as well as of the enhancement of the process of uncoupling of the oxidative phosphorylation [5, 11, 13, 15]. The endoplasmic network is characterized by a sharp reduction in the area and perimeter upon lowering of the rectal temperature from 15 C to 9 °C and by a small difference between the RTs of 9 °C and 6 °C. The changes in lipids are characterized by an increase in their areas at the RT of 6 °C, and the differences in the morphometric parameters of lipids at the RT of 15 and 9 °C are nearly missing.

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THE EFFECT OF CONCENTRATION OF THE INTRACELLULAR HEMOGLOBIN ON THE PERMEABILITY OF RED BLOOD CELLS

There exist two theories of cell permeability: membrane and sorption ones [1]. The membrane theory became very popular due to its very good theoretical and experimental substantiation, while a sorption theory doesn't have thorough experimental grounds. For example, the effect of the concentration of the intracellular content on the cell permeability has not been studied yet. In this respect the authors conducted experimental investigation of the study of RBC permeability under various concentrations of the intracellular hemoglobin. The RBC permeability was studied using a method of EPR spin probe, as described earlier [2]. The factor of permeability (P) was determined on the basis of the diffusion law. The experiments were conducted with human donor blood RBC, stored for 3–4 days at 4 °C. Prior to experimentation the RBC were washed 3 times with a physiological saline of sodium chloride, prepared on 5 mmol/l sodium-phosphate buffer, *pH* 7.4. Pink ghosts of RBC were obtained with an earlier described method [3]. The content of hemoglobin in pink ghosts of RBC was (23.9±1.7)% from the original level. Fig. 1 shows the kinetics of penetration of 20%-glycerol into intact RBC at 0 °C. The permeability factor, calculated from the graphical data (Fig. 2) was equal to (8.1±0.3) x 10^{-6} cm/min. The kinetics of penetration of 20%-glycerol into the pink ghosts of RBC at 0 °C is shown in Fig.3 (curve *1*). It follows from it that the process of glycerol penetration is completed within 4–5 min, while for intact RBC this time exceeded 30 min. The factor of permeability for pink ghosts of RBC was (5.5 ±0.4) x 10^{-5} cm/min. For investigating the influence of a rise in the hemoglobin concentration inside RBC (dehydration) on their permeability the intact RBC were placed into a hypertonic solution of sodium chloride (0.4 mmol/l) in a 1:10 ratio, and the supernatant was removed after sedimentation of RBC by centrifugation. Then a solution, containing 0.4 mol/l sodium chloride, 20%-glycerol, was added to erythromass in a 1:1 ratio, and the kinetics of

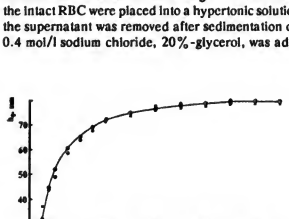

Fig. 1. Kinetics of penetration of 20%-glycerol into RBC at 0 °C (3 exp.)

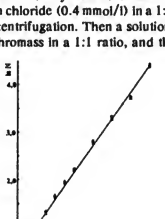

Fig. 2. A plot for calculating the constant of the rate of penetration of glycerol at 0 °C. $N=\frac{h_0-h_t}{h_o}$; h_o – finite value of h; h_t – value of h at the moment of time t

glycerol penetration was investigated (Fig. 3, curve *2*). It follows from it that the kinetics of penetration of glycerol is not registered. A supplementary study using a method of ^{1}H-NMR spectroscopy showed that the glycerol concentration in erythromass by the 20th min was different from that determined by the 2nd minute by 8% (error in determining the concentration value is ±10%). For excluding the action of the electrolyte concentration on the permeability of the RBC membrane the authors conducted experiments with pink RBC ghosts with addition of 0.4 mol/l sodium chloride, as it was in the case of intact RBC (Fig. 3, curve *2*). It follows from this figure that the kinetics of glycerol permeation is observed well enough. The obtained value of the permeability factor is $P=(4.2\pm0.1)\times10^{-3}$ cm/min. This result indicates that one of the reasons of blocking the RBC permeability to glycerol consists not in the changes in the membrane under the action of electrolytes, but in a rise in the hemoglobin concentration due to dehydration. An increase in the hemoglobin concentration in pink ghosts reduces *P* by 23% as compared with the ghosts in the physiological saline, which is sufficiently close to a rise in the microviscosity of the cytosol (15%). A decrease in hemoglobin in RBC results in a more than 6-fold rise in *P*. The results of investigating the effect of the intracellular protein on the permeability of RBC are important not only for understanding the mechanism of regulation of cell permeability, the processes, occurring during cryopreservation of biological systems, but also for the development of new cryobiological technologies.

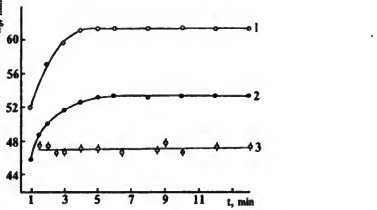

Fig. 3. Kinetics of penetration of 20%-glycerol at 0 °C: *1* – pink RBC ghosts; *2* – pink RBC ghosts in the presence of 0.4 mol/l sodium chloride; *3* – intact RBC in the presence of 0.4 mol/l sodium chloride

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THE EFFECT OF INCREASING CONCENTRATIONS OF NaCl IN THE HYPERTONIC SUCROSE MEDIUM ON THE COLD AND HYPERTONIC SHOCK OF RED BLOOD CELLS

A parallel investigation of the cold and hypertonic shock of RBC showed that blocking of the processes by the inhibitor of the anion transport diisocyanostylbene disulphonate (DIDS) may be of varying extent [2]. The given inhibitor is responsible not only for blocking the anion exchange [6] and release of potassium cations during osmotic stress [3], but it also modifies the interaction of the band 3 protein with the cytoskeletal proteins [5]. It was found that hypertonic stress is determined only by the osmolarity of the pre-incubation medium and is independent of its ionic composition [1], which is responsible for the loss of potassium cations in the hypertonic medium [3]. The above data indicate that the loss of potassium cations may not significantly affect the susceptibility of RBC to hypertonic stress. Considering the above the authors made an attempt to differentiate functional and structural action of DIDS due to the RBC treatment by parachlormercury benzoate (PCMB), during which the interaction of the band 3 protein with the cytoskeleton is broken [4]. The data obtained showed that treatment of RBC by PCMB did not result in considerable changes in the levels of cold and hypertonic stresses. However, the action of the anion transport inhibitor DIDS, which occurs in the nonmodified RBC [2], is almost completely eliminated during treatment of RBC by PCMB, with the exclusion of an incomplete reduction of the effect of DIDS during cold shock (fig. 1). This phenomenon may be interpreted in such a way that the action of DIDS on the hypertonic stress relates, mainly, to the modification of the interaction of the band 3 protein with the cytoskeleton. At the same time a residual blocking effect of DIDS on the cold shock (Fig. 1, curve *3*) may occur due to the inhibition of the loss of potassium cations [3]. In this respect it may be assumed that a

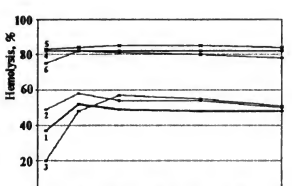

Fig. 1. The effect of treatment by PCMB on the susceptibility of RBC to cooling down to 0 °C (*1, 2, 3*) and hypertonic stress after transfer into 3 mol/l NaCl (*4, 5, 6*) after incubation at 37 °C in the medium, containing 0.86 mol/l sucrose: *1, 4* – control, *2, 5* – PCMB, *3, 6* – PCMB+DIDS

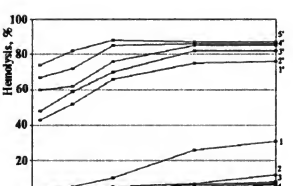

Fig. 2. Effect of the increasing concentrations of NaCl on the susceptibility of RBC to cooling down to 0 °C (*1, 2, 3, 4, 5*) and hypertonic stress after transfer into 3 mol/l NaCl (*1', 2', 3', 4', 5'*) after incubation at 37 °C in the medium, containing 0.7 mol/l sucrose: *1, 1'* – 0 mmol/l NaCl; *2, 2'* – 25; *3, 3'* – 50; *4, 4'* – 100; *5, 5'* – 150

method of combining the pre-incubation medium with the increasing concentrations of NaCl should result in a rise in the susceptibility of RBC to hypertonic stress, reducing their susceptibility to cooling. In this respect the authors studied the effect of the increasing concentrations of NaCl on the susceptibility of RBC to cooling and hypertonic stress after incubation in the medium, containing 0.7 mol/l sucrose. An increase in the NaCl concentration in the incubation medium up to 150 mol/l results in a rise in the susceptibility of RBC to transfer into 3 mol/NaCl, however, the susceptibility of cells to cooling is thus reduced (Fig. 2). Thus, the results obtained testify to the fact that susceptibility of red blood cells to hypertonic stress is not determined by a release of potassium cations. Besides, the impairment of the interaction of the band 3 protein with the cytoskeleton does bring about a loss of the cold and osmotic susceptibility of the cells.

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APPLICATION OF A PENNING METHOD FOR HARVESTING STEM-CELLS ENRICHED POPULATIONS FROM CRYOPRESERVED BONE MARROW

Application of monoclonal antibodies (MAB) in modern immunohematologic investigations permits to identify a wide spectrum of membrane structures on the cells with one or other functional properties, to reveal the role of these structures. With the help of MAB it is possible to eliminate various subpopulations of cells, e.g. hemopoietic precursors, from a total heterogeneous population of the bone marrow (including cryopreserved), increasing their concentration. The enriched fractions of hemopoietic precursors without a large number of factor-producing cells may be used for studying the role of every population and recombinant exogenous growth factors in the regulation of hemopoiesis. Population of hemopoietic precursors

may be also applied in transplantation of allogenic bone marrow, when it is necessary to reduce the risk of the development of immune conflicts, considering a possibility of development of immunologic tolerance in the immunocompetent cells, which are formed out of them. Besides, application of such a methodical approach makes it possible to evaluate survival, possible modification of membrane structures after exposure of the hemopoietic cells to the action of cryopreservation factors, to give assessment of the concentration parameters of these cells in the total population. Currently available methods of selection of stem cells make it possible to achieve a high degree of purification, however, the most effective among them, e.g. involving cell sorters, are very expensive, which limits a possibility of their application. On the other hand, treatment of the bone marrow suspension in a cytotoxic test (by MAB and complement) does not allow to obtain both fractions being separated in viable states. In the investigations conducted use was made of a direct penning-method with MAB to Thy-1.2 antigen, based on the ability of the Thy-1.2$^+$ cells to be sorpted in the cells of plastic case, covered by MAB [2]. Isolation of the sorpted Thy-1.2$^+$ cells was done by soft pippetting with subsequent administration to the lethally irradiated mice for evaluation of the colony-forming activity of the isolated population [4]. Statistical processing of the data was performed using a Student-Fisher method. The experiments conducted showed that after sorption on MAB from the bone marrow of intact donors about 2.5% of Thy-1.2$^+$ cells are eliminated (fraction C-1), while after a repeated sorption – about 0.5% of Thy-1.2$^+$(fraction C-2), which correlates with the data reported by other researchers [3]. Fraction C-1 possessed a colony-forming activity, which was by 3.5-fold higher as compared with control, by the 8th day, and by 10-fold – by the 12th day (Table 1). Apparently, a colony-forming activity of the fractions, sorpted on MAB, was due to the availability of Thy-1.2$^+$CFUs in them, and a Thy-1.2$^+$ fraction is a population, enriched with hemopoietic precursors of various extent of differentiation. It is evident from a comparison of a concentration of CFUs in the original suspension and that sorpted on MAB, followed by simple mathematical processing [1], that the content of Thy-1.2$^+$ CFUs-8 in the bone marrow does not exceed 5–10% from the total amount of CFUs-8, while Thy-1.2$^+$–12 comprise approximately 30–35% of all CFUs-12. The data, submitted in Table 1, testify to the fact that sorption on MAB of the cells of the bone marrow, which is regenerated after a cytostatic treatment, cannot reflect the ratio of hemopoietic cells in such a myelotransplant.

Colony-forming activity of various fractions of bone marrow: intact, cryopreserved and treated with 5-FU

Fractions of the transplanted bone marrow	Transplant					
	Intact native, 1×10^5/mice		Treated with 5-FU, 5×10^3/mice		Intact cryopreserved, 1×10^5/mice	
	CFU_s-8	CFU_s-12	CFU_s-8	CFU_s-12	K-1 CFU_s-12	K-2 CFU_s-12
Original control	14.2±1.5°	16.4±0.9	14.4±2.6	16.9±2.9	13.5±1.7	11.2±2.0
C-1	51.1±8.1*	163.3±12.0*	1.8±0.6*	32.4±4.1*	122.9±12.3*	27.3±9.1*
C-2	29.4±5.2*	56.0±6.3*	3.6±2.4*	20.3±3.8*	-	-

Note: the experiments were conducted with mice of a CBA line; 380 animals were used in experiments; ° – average value for 9 spleens; * – significant differences ($p < 0.05$) as compared with control.

In 8 days after injection of fluorouracyl (5-FU) at a dose of 150 mg/kg body weight against a rapid reduction in the content of the bone marrow nucleated cells (by 10-12-fold) the absolute content of CFUs-12 and CFUs-8 in the femur exceeded their levels in the normal bone marrow by nearly 20-fold. From this suspension with the help of penning method about 5% Thy-1.2$^+$ cells were eliminated. However, in spite of an increasing content of CFUs and Thy-1.2$^+$cells as whole in such a bone marrow, the concentrations of Thy-1.2$^+$-CFUs-8 among all the CFUs-8 cells decreased down to 0.5-0.6%, i.e. it was by 16-18-fold lower, as compared with the population of CFUs-8 of the normal bone marrow. The concentration of CFUs-12 in a C-1 fraction was approximately by 2-fold higher as compared with the whole bone marrow, treated with 5-FU and, subsequently, was by more than 40-fold higher in comparison with the normal bone marrow. Despite this, the content of Thy-1.2$^+$-CFUs-12 was about 15% from the whole CFUs-12, i.e. it was by 2-fold lower, as compared with the normal bone marrow, which may be a consequence of dilution of Thy-1.2$^+$CFUs, remaining in the bone marrow of the femur bone, by the newly formed Thy-1.2$^+$CFUs, since a reduction in the portion of Thy-1.2$^+$CFUs by 2-fold became evident after a 2-fold rise in the total amount of CFUs-12 in the bone marrow of the femur bone. After arousing from the state of profound cold anabiosis the indices, characterizing not only functional, but also structural properties of these cells, are altered significantly [1]. Depending on the selected mode of cryopreservation the amount of colonies, formed by a cryopreserved bone marrow, may vary: cryopreservation with a 10%-solution of DMSO (K-1) provides for a high survival of CFUs (90%). The number of colonies, formed by a cryopreserved bone marrow under protection of a 7.5%-PEO (K-2), was reduced by 35%. It follows from the results obtained, that cryopreservation in a K-2 mode is followed by the impairment/alteration of the state of Thy-1.2 structures, which is supported by a manifested decrease in the total amount of cells, sorpted on MAB, which comprises not more that 15% from the native or a K-1 cryopreserved bone marrow. The calculations showed that the ability of Thy-1.2$^+$CFUs to interact with MAB after cryopreservation (K-2) was reduced even to a larger extent as compared with the whole fraction of Thy-12.++ cells, thus comprising not more than 5-6% from the native control. However, in the given model penning-method provided for enrichment of the myelotransplant by CFUs by 2-5-fold. The results obtained support a possibility of applying pen-

ning-method for evaluating qualitative state of the cryopreserved myelotransplant, namely, for identifying the availability of the receptor structures on hemopoietic cells, and testify to the applicability of this method for harvesting fractions of the cryopreserved myelotransplant, enriched with hemopoietic precursors.

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ACTIVITY OF THE ENZYMATIC ANTIOXIDATIVE SYSTEM OF THE RAT LIVER AFTER ACUTE COOLING OF THE ORGANISM

In the experiments on studying the intensity of lipid peroxidation (LPO) in the time course of self-warming after acute cooling down to the state of profound hypothermia (RT of 19–20 °C) the liver homogenates of experimental animals demonstrated phase changes in the accumulation of malone dialdehyde. The maximums of a rise in the LPO level were observed in 3 and 24 hours after cold exposure. One of a possible explanations of the LPO activation may be a reduction in the activity of a glutathione-dependent enzymatic system of the cells. This is confirmed by a change in the content of the reduced glytathione (GSH) in the hepatic tissue (a reduction by approximately 26–27% in 3 and 24 hours of self-warming), observed in the same series of experiments, which was also of a phase-like nature, though its direction was opposite to the changes in the LPO intensity. Since basic enzymes, utilizing hydroperoxides of the unsaturated fatty acids, are NADP-dependent, a rise in the LPO intensity may occur due to a reduction in the supply of the protective antioxidative system of the cell with the reducing equivalents. Judging from the above, with the aim of revealing more subtle mechanisms of the LPO level regulation in the warm-blooded organism during a post-cold period the author decided to conduct a study of the activity of a glutathione-dependent antioxidative system and the system of NADH recovery. In the given series of experiments male Wistar rats with the body weights of 200–220 g were cooled in the process of free swimming in water with the temperature of 7 °C down to the RT of 19–20 °C. Slaughtering of experimental animals was done in 3 and 24 hours after self-warming. Intact rats were used as control. In the liver homogenates, prepared on 100 mM tris-HCl buffer (*pH* 7.4, a tissue/buffer ratio was 1:3), the author determined glutathione peroxidative, with H_2O_2 and with cumole hydroperoxide (GSH-peroxidase), glutathione-S-transferase (GSH-S-transferase) and glutathione-reductase (GSH-reductase) activities [2]. The rate of $NADP^+$ restoration in the same homogenates was judged upon by the activity of malate dehydrogenase (MDG) [3], glucoso-6-phosphate-dehydrogenase (G-6P-DG), 6-phospho-gluconate-dehydrogenase (6P-G-DG) [4] and isocitrate-dehydrogenase (Ic-DG) [5].

Table 1

Activities of some GSH-dependent antioxidative enzymes (nmol substrate/1 mg protein/1 min) in the liver homogenates of rats after acute cooling (Sx±x; *n*=5-7)

Enzymatic activity	Time of self-warming, h		
	Control	3	24
GSH-peroxidative with H_2O_2	49,9±5,4	36,4±1,8*	32,7±1,2**
GSH-peroxidative with cumole hydroperoxide	213,6±11,3	194,8±13,2	180,6±30,2
GSH-S-transferase	719,8±54,5	753,9±45,5	671,8±79,8
GSH-reductase	44,9±4,8	56,7±1,03**	58,5±5,2*

Note: * – $p < 0.1$; ** – $p < 0.05$ as compared with control.

Less pronounced changes occurred under the given experimental conditions in the system of $NADP^+$ recovery (Table 2). Among the investigated enzymes the author observed only a tendency towards reducing (p) the activity of Ic-DG, while the values of activity of MDG, G-6P-DG, 6P-G-DG did not differ from the indices, registered in control animals. The results obtained nicely correlate with the data published elsewhere. For example, some authors [1] report on the tendency towards reduction in the peroxidative activity in 24 hours and a rise in the glutathione-reductase activity in 2 hours after exposure

with subsequent decrease by the 24th hour in the livers of the rats, exposed to low temperatures in cold chamber at – 12+27 °C for 4 hours (RT remained unchanged). These authors believe that activation of GSH-reductase at the initial stages of cold exposure is a compensatory response to a reduction in the content of GSH in the cell. A decrease in its activity at the relatively remote stages of self-warming is indicative of the insolvency of this system of regeneration of recovered glutathione.

Table 2

Activity of NADPH-dependent dehydrogenases (nmol NADP/1 mg of protein/1 min) in the liver homogenates of rats after acute cooling

Enzymatic activity	Time of self-warming, hours		
	Control	3	24
MDG	18.3±2.1	18.2±0.8	15.8±2.2
6P-G-DG	8.1±1.0	10.1±2.9	9.5±1.5
G-6P-DG	13.4±1.1	13.9±4.5	12.1±4.0
Ic-DG	226.0±32.6	157.0±15.7*	173.5±29.7

Note: * – $p < 0.1$ as compared with control.

Thus, considering the fact on the absence of pronounced changes in the activity of $NADP^+$-reducing enzymes, as well as taking into account a considerable reduction in the content of reduced glutathione in the liver tissue under the given experimental conditions, it may be assumed that the weakest link in the glutathione-dependent system of utilizing hydroperoxides of the unsaturated fatty acids is reparation of glutathione. Nevertheless, a probable depletion of glutathione-dependent antioxidative system, which occurs due to the insufficient rate of NADP recovery, cannot be excluded. Further detailed studies are required to answer this question.

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SUSCEPTIBILITY OF SUBPOPULATIONS OF RED BLOOD CELLS OF DIFFERENT AGE TO POSTHYPERTONIC HEMOLYSIS

A model of RBC injury during slow freeze-thawing may be a process of their posthypertonic lysis [6]. Transfer of RBC from hypertonic into isotonic medium results in the injury of their erythrocytic membrane and lysis, which was termed as posthypertonic. The data reported elsewhere [5, 9] support an important role of the composition of the hypertonic and isotonic solutions, time of exposure of RBC in them and original tonicity, after which the cells were exposed to hypertonic treatment by means of dialysis [7] or freezing and subsequent thawing [5]. In the course of PL the average volumes of the cells are increased as compared with the physiological value, however, the rehydrated cells respond differently, since the curves of the volume distributions testify to the existence of two subpopulations of RBC: swollen and those which remained normal [8]. Such a behaviour of the systems corresponds to the response of the type «all or nothing», which is known for certain phenomena, when a selectively acting factor is unequally distributed among the cell in the whole population. Varying degree of the RBC susceptibility to dehydration is supported by the fact that experimental treatment, modelling the processes of freeze-thawing, results in hemolysis of only part of the cells [5-9]. The given reasearch deals with the study of a hypothesis, according to which different resistance of RBC in the course of PL may relate to the inner heterogeneity of the total population of cells, which is manifested in different value of their density, which correlates with the age of cells [1, 2]. The donor blood RBC were thrice washed in physiological saline (10 mM *tris*-HCl, 150 mM NaCl, *pH* 7.4) and fractionated in a multi-stge Ficoll gradient (Ficoll-400, Pharmacia). Ficoll was dissolved in physiological saline, and the densities of thus prepared solutions were in the range of 1.04 to 1.12 g/cm^3; 6 ml of these solutions were placed layer-by-layer in a centrifuged vial for obtaining 4 stages of a Ficoll gradient, and 1.6 ml of a carefully dissolved RBC suspension were placed on top. Centrifugation took place at 350 g for 4 min at room temperature. The obtained subpopulations of RBC of varying density

Table 1

Parameters of the eryhtrocytic fractions, obtained during centrifugation in Ficoll gradient

Fraction number	K/K_o	Hb/Hb_o	V/V_o	Number of cells in the fraction, %
TEM	1	1	1	100
1	1.21±0.08	1.1±0.08	1.01±0.01	33.2
2	0.91±0.1	0.98±0.02	0.99±0.01	55.6
3	1.08±0.11	1.10±0.07	1.00±0.01	12.7
4	0.85±0.12	0.88±0.07	0.98±0.01	1.5

Note: the content of the K-ions (K), hemoglobin (Hb), average cell volume (V) are given with respect to the corresponding parameters in TEM.

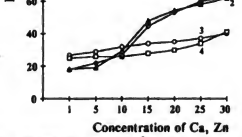
were taken from every layer of the gradient and twice washed in physiological saline. Ten μl of suspension of every subpopulation were placed into distilled water or cyan methemoglobin reagent for determining, respectively, the content of K^+ ions or hemoglobin in the cells. Testing the susceptibility to PL involved by exposing 10 μl of RBC suspension in 1 ml of the hypertonic sucrose solution (1.2 M) or NaCl (1.5 M), pre-heated up to 37C or cooled down to 0C, for 15 min at 37C and 90 min at 0C. Then 10 μl of the cell suspension were tranferred into 1 ml of the isotonic sucrose or NaCl in the presence or absence of the cations Zn^{2+} and Ca^{2+} with subsequent exposure for 5 min at room temperature. The obtained samples were centrifuged at 600 g for 3 min. After that the extent of the cell hemolysis was determined spectrophotometrically with a wavelength of 415 nm. The average volumes of the cells of the total erythrocytic mass (TEM) and subpopulations were measured with a Coulter-counter with the sensor orifice of 50 μm and the length of 50 μm, using a current of less than 0.3 mA. Centrifugation in the multistage Ficoll gradient permitted to obtain 4 fractions of RBC. Table 1 shows some parameters of the cell fractions, measured as the relative units in comparison with the corresponding values of the non-fractionated blood.

Table 2

Level of PL (%) after incubating TEM and fractions in the hypertonic solutions of NaCl and sucrose for 15 min at 37 °C and 90 min at 0 °C and subsequent rehydration in the corresponding isotonic medium

Fraction number	Posthypertonic lysis, %			
	1.2 M sucrose		1.5 M NaCl	
	0 °C	37 °C	0 °C	37 °C
TEM	2	24	15	85
1		20		79
2		24		84
3		25		86
4		24		83

Though erythrocytic fractions were of different densities, the values of the parameters were similar, with a tendency towards increasing the volume, the content of K^+ and hemoglobin in fraction 1, and reduction of the features in the last fraction. These data correlate with the other results, where Ficoll and Percoll were used as the gradient carriers [1]. Table 2 shows the proportion of hemolysis after incubating RBC in the hypertonic solution and transferring them into isotonic conditions. Equillbration of cells in the NaCl solution is characterized by a higher proportion of hemolysis as compared with the sucrose medium, however, this effect was also evident both in control and fractionated samples, and the extents of the RBC hemolysis were similar to every medium with a minor tendency of reducing hemolysis in fraction 1. Lowering of the temperature of the hypertonic medium down to 0C the level of PL was significantly reduced, despite prolongation of the time of exposure up to 90 min. the experiments conducted showed that the factor of temperature much higher determined the RBC susceptibility to posthypertonic injury in contrast with the criterion of cell separation in Ficoll gradient. It is known that divalent cations Ca^{2+} and Zn^{2+} significantly affect the extent of PL [8]. Fig. 1 shows the dependence of the level of PL, obtained for fraction 3 and non-fractionated blood, on the co-concentrations of the ions Ca^{2+} and Zn^{2+} in the rehydration medium. A rise in the concentration of Ca^{2+} stimulates PL of the cells, dehydrated in the hypertonic sucrose solution, but it exercises an opposite action on the cells, incubated in the hypertonic NaCl. The ions Zn^{2+} produce a similar activation effect on Pl of the cells, dehydrated in sucrose, and do not affect PL of the cells, treated with the hypertonic NaCl. From the first glance these data contradict to those obtained earlier, when PL of RBC was inhibited by the same ions [8]. It was found, however, that the direction of action of Ca^{2+} and Zn^{2+} is dependent on the following factor: whether the ion is present in the rehydration medium from the very beginning or it is added to it after the cells are transferred into the medium. Thus, a PL-activating influence of cations Zn^{2+} and Ca^{2+} was used for determining the nature of the injury to RBC from various subpopulations during dehydration-rehydration. It was also found that non-fractionated



Effect of divalent cations Ca^{2+} mM (1, 2) and Zn^{2+} μM (3, 4) on the posthypertonic lysis of total erythrocytic mass (2, 3) and third fraction (1, 4). The RBC were incubated for 15 min in hypertonic solutions of NaCl (closed symbols) and sucrose (open symbols) and rehydrated in the corresponding isotonic solutions in the presence of the above concentrations of divalent cations at room temperature

blood and all the subpopulations demonstrated similar reactivity towards the action of cations, as it is shown in Fig. 1 for the third fraction. Dehydration at 0C not only significantly reduces the portion of PL, but also eliminates the activating and inhibiting action of divalent cations. Neither Ca^{2+}, nor Zn^{2+} under the given conditions change the level of PL under the given conditions as compared with the control level (the data are not submitted). Hence, the results obtained demonstrated that susceptibility of RBC to PL is not determined by the parameters, according to which they are separated in the density gradient. Ir other words, every cell fraction contains approximately equal amount of cells, which are potentially capable for swelling and lysis after their transfer from the hypertonic into the isotonic medium. The type of treatment (electrolyte content of the dehydrating medium and temperature) is the most important for alteration of the RBC properties in the process of PL. It is known that a long-term deformation of a membrane-skeleton complex results in the shift of a dimer-tetramer equilibrium of spectrin towards formation of oligomers, and this process is of a manifested temperature dependence [3]. In fact, cell exposure to hypertonic conditions corresponds to a similar membrane deformation. Besides, a rise in the concentration of hemoglobin and cytoskeletal proteins in the dehydrated RBC facilitates oligomerization of spectrin [4]. Lowering of temperature and shortening of incubation reduce both the ability of spectrin for self-association [3, 4] and PL. Apparently, «prolytic» changes in the interactions of the cytoskeletal-membrane protein components of RBC are responsible for varying susceptibility of cells of the total erythrocytic mass to PL.

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Проводится подписка на журнал "Проблемы криобиологии". С 1985 г. научно-теоретическое издание выходило под названием "Криобиология", с 1991 г. – "Проблемы криобиологии". Издается на русском и английском языках. Объем 5,6 печ. л., периодичность четыре номера в год. В журнале публикуются научные работы по всем аспектам действия охлаждения и замораживания в биологии, медицине и пищевой промышленности, по проблемам гипотермии, низкотемпературному консервированию клеток и тканей, криохирургии, по сублимационному высушиванию, о криопротекторных веществах и их фармакологическом действии. Освещаются вопросы гибернации, зимостойкости растений, конструирования приборов и оборудования для низкотемпературного консервирования биологических объектов и криомедицины.

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